BIOCHEMICAL AND MOLECULAR BASIS FOR THE PRO- ATHEROGENIC PROPERTY OF DYSFUNCTIONAL HIGH DENSITY LIPOPROTEIN

A THESIS PRESENTED BY

SINI. S

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2016

CERTIFICATE

I, **Sini.S,** hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Biochemical and Molecular Basis for the Pro-atherogenic Property of Dysfunctional High Density Lipoprotein".** No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Signature 

Date **30-06-2016**

Sini.S

Dr. N. Jayakumari
Professor
Department of Biochemistry

CERTIFICATE

This is to certify that Ms.Sini. 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, Trivandrum. The thesis entitled **"Biochemical and Molecular Basis for the Pro-atherogenic Property of Dysfunctional High Density Lipoprotein"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Signature 

N. Jayakumari

Date 30.06.2016

The thesis entitled

BIOCHEMICAL AND MOLECULAR BASIS FOR THE PRO-ATHEROGENIC PROPERTY OF DYSFUNCTIONAL HIGH DENSITY LIPOPROTEIN

Submitted by

SINI. S

For the degree of
Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
Thiruvananthapuram

Is evaluated and approved by

Name of the guide
N. Jayakumari

Name of the thesis examiner
P.R. Sridhakaran

Acknowledgements

I consider myself privileged to have had the opportunity to carry out my doctoral studies in the Department of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, India. I thank Dr. K Radhakrishnan, Dr. Jaganmohan Tharakan, the former directors and Dr. Asha Kishore, the present Director of SCTIMST, for extending support and excellent facilities required for research programs in this institute. I owe a great gratitude to Registrar, Dr.A.V.George and to Deputy registrar, Dr.Sundar Jayasingh for their dedicated support in carrying out my PhD work in SCTIMST. I acknowledge the financial support received from INSPIRE, Department of Science and Technology, India.

I owe a huge depth of gratitude to my mentor, Dr. N. Jayakumari for her continued encouragement and constant support in my research program, which has helped me greatly in the successful completion of my studies. Not a single part of the work would have materialized without the constant support, unfailing encouragement and practical suggestion of my guide. I consider it as a great opportunity to do my doctoral programme under her guidance and to learn from her research expertise.

I express my sincere thanks to Dr. P.S Appukuttan, Head of the Dept. of Biochemistry for his encouragement and advice. I am thankful to Dr. Srinivas. G, for his motivation and valuable suggestions which help me to built-up my work in a fruitful way. I am indebted to all my colleques, senior and junior staff of my department for constructive discussion and among them I want to mention Dr.Deepa, Dr.Geetha.M and Ms.Sumitha for their guidance and valuable suggestions during the course of my work.

I warmly thank Dr.R.Renuka Nair and Dr. S.Harikrishnan, the members of my Doctoral Advisory Committee for their guidance and helpful suggestions. Iam thankful to Dr.K. Shivkumar for his valuable suggestions.

I am thankful to all staff of the Dept. of Blood Transfusion Services and Central Clinic Laboratory of this Institute for providing me the blood samples at any time all throughout the duration of my study.

I owe a huge depth of gratitude to my UG course HOD, Dr. K.G. Padmakumar, for his support and valuable suggestions in my educational life.

I am very greatful to Mr.Ranjith Divakaran, Biomedical Engineer, B.M.T Wing, for his timely support during my joining procedures in SCTIMST.

My sincere thanks to Department of computational biology and Bioinformatics, Kerala University and Biogenix research centre, Trivandrum for helping me to carry out the analysis.

I appreciate the goodwill extended to me by all my friends and a word of thanks would be far too inadequate to express the gratitude I have for them. I am extremely fortunate to have a loving family that was a pillar of strength and support to me at all occasions, which has been crucial in the progress of my studies. Above all, I owe it to the Lord Almighty for granting me this opportunity and enabling me to achieve this goal.

Sini.S

TABLE OF CONTENTS

Declaration of the student……i

Certificate of the guide……ii

Approval of the thesis……iii

Acknowledgements……...iv-v

List of figures……xii-xiv

List of tables……xv

Abbreviations…… xvi-xviii

Synopsis…… xix- xxvi

1.1. INTRODUCTION 1

1.2. Pathogenesis of coronary heart disease..... 1

1.3. The role of HDL in atherosclerosis..... 2

1.4. Significance of the study 3

Objectives of the study 5

2. REVIEW OF LITERATURE 6

2.1. Cardiovascular diseases..... 6

2.2. Atherosclerosis and Coronary heart disease 7

2.3. Coronary heart disease scenario in India 8

2.4. Risk factors for coronary heart disease 10

2.5. Pathogenesis of atherosclerotic -CHD 15

2.6. Current theories of atherosclerosis 20

2.6.1. The Response-To-Injury Hypothesis of Atherosclerosis.... 21

2.6.2. Endothelial dysfunction and atherosclerotic risk 21

2.7. Inflammation and oxidative stress in atherosclerosis 24

2.7.1. Inflammation and atherosclerosis 24

2.7.2. Role of Matrix metalloproteinases in atherosclerosis 28

2.7.3. Oxidative stress and atherosclerosis 31
2.8. Role of Lipoproteins in Atherogenesis 34
2.8.1. Modified low-density lipoproteins and atherosclerosis..... 36
2.8.2. HDL and atherosclerosis 39
2.8.3. HDL structure, composition and metabolism 41
2.9. Antiatherogenic functions of HDL 48
2.9.1. HDL and Reverse Cholesterol Transport(RCT) pathway .. 49
2.9.1.1. RCT and involvement of macrophage receptors..... 52
2.9.2. Antioxidant and anti-inflammatory properties of HDL..... 54
2.9.3. Antiapoptotic property of HDL 56
2.10. HDL-raising therapies 57
2.11. Functionally modified HDL and atherosclerosis..... 58
3. MATERIALS AND METHODS..... 63
3.1. Materials 63
3.2. Methods 64
3.2.1. Blood sample collection 64
3.2.2. Analytical methods 65
3.2.2.1. Estimation of total cholesterol in serum 65
3.2.2.2. Estimation of triglycerides in serum..... 66
3.2.2.3. Quantitation of LDL-C in serum 66
3.2.3. Isolation of HDL (d= 1.063- 1.21) by ultracentrifugation... 67
3.2.3.1. Isolation of HDL by PEG and estimation of HDL-Cholesterol..... 67
3.2.3.2. Electro elution of HDL 68
3.2.4. Isolation of LDL(d= 1.019-1.063) by ultracentrifugation .. 68
3.2.4.1. Assay of total proteins..... 69

3.2.4.2. Polyacrylamide disc gel electrophoresis (PAGE) of isolated lipoprotein 70
3.2.4.3. Assay of antioxidant capacity of HDL (Functional assay) 71
3.2.4.4. Assay of serum lipidperoxides (TBARS assay) 72
3.2.4.5. Assay of serum protein carbonyls 73
3.2.4.6. Assay of serum paraoxonase activity [PON-1] 73
3.2.4.7. Assay of Serum hs-CRP 74
3.2.5. Monocyte cell isolation and culture 74
3.2.5.1. Monocyte differentiation to macrophages 75
3.2.5.2. Estimation of cell viability by trypan blue exclusion test 75
3.2.5.3. MTT (4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromide) assay 76
3.2.5.4. Cell treatment 76
3.2.5.5. Measurement of intracellular reactive oxygen species (ROS) 76
3.2.5.6. Assay of tumour necrosis factor- alpha(TNF-α) 77
3.2.5.7. Assay of interlukin 10 (IL-10) 78
3.2.5.8. Activity of gelatinases or matrix metalloproteinases (MMP- 2 & MMP- 9) 78
3.2.5.9. Oil Red O staining for neutral lipid accumulation 79
3.2.5.10. Quantitation of cellular cholesterol 79
3.2.5.11. Propidium iodide staining for measuring cell death 80
3.2.5.12. Estimation of glutathione peroxidase 80
3.2.5.13. Estimation of glutathione reductase 81
3.2.5.14. Estimation of superoxide dismutase (SOD) activity 81
3.2.5.15. Estimation of catalase activity 82

3.2.5.16. Estimation of reduced glutathione 82
3.2.5.17. Comet (Single-cell gel electrophoresis) assay 83
3.2.5.18. Mitochondrial membrane potential by flow cytometry. 84
3.2.5.19. Apoptosis by flow cytometry 86
3.2.5.20. qRT-PCR analysis for CD36 expression 86
3.2.5.21. Western blot analysis 88
3.3. Statistics 89
4. RESULTS 90
4.1. Identification of the prevalence of functionally altered HDL in apparently healthy subjects and in pateints with clinically diagnosed coronary artery disease 90
4.1.1. Isolation of HDL and LDL 91
4.1.2. Purity of isolated lipoprotein 91
4.1.3. Antioxidative capacity of HDL in healthy subjects and in CAD patients 92
4.2. HDL Functionality and Serum Lipid Parameters 95
4.2.1. HDL Functionality and systemic oxidative Stress markers. 97
4.2.2. HDL functionality and systemic inflammation 98
4.2.3. Characterisation of isolated HDL 98
4.3. Association of matrix metalloproteinase 9 (MMP 9) with dysfunctional HDL 100
4.3.1. Association of MMP 9 with dysfunctional HDL 101
4.3.2. HDL subfractions and MMP9 activity 103
4.3.3. Treatment of HDL with MMP9 and functional characterization of HDL 103
4.3.4. Proteolytic activity of MMP 9 on apo A1/HDL..... 105
4.4. Dysfunctional HDL induces proinflammatory response 105
4.4.1. Monocyte isolation and culture 105

4.4.2. cell viability 106
4.4.3. Pro-inflammatory response: 107
4.5. Biomolecular modeling showing binding of MMP9 with HDL 108
4.6. Influence of Particulate matter associated with air pollution (polycyclic aromatic hydrocarbons) on HDL functionality..... 110
4.7. Investigation of the effect of dysfunctional HDL on human monocyte-derived- macrophage function relevant to atherosclerosis 111
4.7.1. Dysfunctional HDL and cholesterol homeostasis in monocyte-macrophages..... 113
4.7.1.1. Human macrophage culture 113
4.7.1.2. Cell treatment 113
4.7.1.3. piHDL induces oxidative stress, lipid accumulation and foam cell formation in human monocyte-derived- macrophages 114
4.7.1.4. Assessment of expression of LOX1, ABCG1, SR-B1 and CD36 in macrophages 115
4.7.1.5. piHDL stimulates ERK1/2 MAPK and NFkB expression 118
4.7.1.6. piHDL induces CD36-dependent activation of ERK1/2 in macrophages 119
4.7.1.7. piHDL activates PPAR-γ and Nrf2 in macrophages .. 122
4.8. Dysfunctional HDL and macrophage apoptosis..... 123
4.8.1. Dysfunctional HDL induces oxidative stress in human monocyte-derived macrophages 123
4.8.2. Sources of intracellular ROS stimulated by dysfunctional HDL…… 125
4.8.3. Antioxidant enzyme status in human monocyte-derived macrophages exposed to piHDL..... 126

4.8.4. Dysfunctional HDL induces lipid accumulation in human monocyte-derived macrophages 127

4.8.5. Dysfunctional HDL induces cytotoxicity in human monocyte-derived macrophages 128

4.8.6. Dysfunctional HDL induces mitochondrial membrane potential ($\Delta\Psi_m$) alteration, DNA fragmentation and apoptosis in human monocyte-derived macrophages..... 129

4.8.7. PARP [poly(ADP-ribose) polymerase] cleavage in macrophages treated with piHDL 132

4.8.8. Dysfunctional HDL and macrophage autophagy 134

5. DISCUSSION 136

5.1. Identification of the prevalence of functionally altered HDL in apparently healthy subjects and in patients with clinically diagnosed coronary artery disease 136

5.2. Association of matrix metalloproteinase-9(MMP-9) with dysfunctional HDL 139

5.3. Investigation of the effect of dysfunctional HDL on human monocyte-derived-macrophage function relevant to atherosclerosis 143

5.4. Dysfunctional HDL and macrophage apoptosis..... 149

SUMMARY AND CONCLUSION..... 154

FUTURE DIRECTIONS 159

6. BIBLIOGRAPHY 160

LIST OF PUBLICATIONS FROM THE THESIS 181

LIST OF FIGURES

Figure No. **Caption** **Page**

Figure 1:Traditional versus nontraditional risk factors for coronary artery disease. 13

Figure 2:Atherosclerosis events occurring in an artery. 20

Figure 3: Structure of HDL..... 45

Figure 4:Anti atherogenic properties of HDL. 49

Figure 5:Reverse cholesterol transport pathway..... 52

Figure 6:Disc gel electrophoretic pattern of serum lipoproteins on 3.75% polyacrylamide gels. 91

Figure 7:Antioxidant capacity of HDL from healthy subjects[nHDL] and piHDL against LDL-oxidation. 92

Figure 8: Inflammatory index of HDL calculated using the fluorescence of oxidized LDL as an index.....94

Figure 9: Antioxidative capacity of HDL isolated from healthy volunteers. 95

Figure 10:Lipid profile of subjects having functional and dysfunctional HDL..... 96

Figure 11:Systemic oxidative stress status of subjects having functional and dysfunctional HDL. 97

Figure 12: Systemic inflammatory status of subjects having functional and dysfunctional HDL……98

Figure 13: MMP 9 activity in isolated lipoproteins:..... 101

Figure 14:MMP 9 activity in HDL fractions isolated by three different methods and corresponding functionality of HDL..... 102

Figure 15: MMP 9 activity in HDL subfractions..... 103

Figure 16:MMP 9 activity in HDL fraction pre-incubated with MMP9 and functionality of HDL. 104
Figure 17: Proteolytic activity of MMP 9 on Apo A1 105
Figure 18: The CD14+ cells(monocytes). 106
Figure 19:Cell viability by MTTassay. 107
Figure 20:Monocyte/macrophage cytokine release induced by HDL. 108
Figure 21:Docked structure showing interaction between HDL and MMP 9..... 110
Figure 22:Effect of PAH components on HDL functionality. 111
Figure 23:CD68+ cells(macrophages). 113
Figure 24:Dysfunctional HDL induces ROS production and lipid accumulation in human monocyte-derived macrophages. . 115
Figure 25:Macrophage receptor expression following exposure to HDL. 117
Figure 26:Influence of piHDL and nHDL on MAPKs and NFkB expression. 119
Figure 27:Cross- talk between CD 36 and ERK1/2 expression in macrophages following exposure to piHDL. 121
Figure 28:Influence of piHDL on expression of PPAR -γ and Nrf2 in macrophages. 122
Figure 29:Time- dependent ROS production in macrophages. 124
Figure 30:Sources of intracellular ROS induced by piHDL in macrophages. 126
Figure 31:Time- dependent cellular cholesterol accumulation induced by piHDL..... 128
Figure 32:Dysfunctional HDL induced cell death in human macrophages. 129

Figure 33:Mitochondrial membrane potential ($\Delta\Psi_m$)alteration, and DNA fragmentation in macrophages in response to piHDL..... 131
Figure 34:PARP cleavage in macrophages in response to piHDL..... 132
Figure 35:Benzamide inhibition of PARP cleavage in macrophage apoptosis..... 133
Figure 36:Autophagosomal marker- LC3-1 and LC3-II expression in macrophages..... 134

LIST OF TABLES

Table No.	Caption	Page
Table 1:	Basic characteristics of study subjects	90
Table II:	Characterization of isolated HDL particle	99
Table III:	Effect of piHDL on macrophage antioxidant status	127

ABBREVIATIONS

7-AAD	-	7-Amino actinomycin D
ABCA1	-	ATP –binding cassette transporter A1
ABCG1	-	ATP –binding cassette transporter G1
AP-1	-	Activation protein 1
APR	-	Acute phase response
ATP	-	Adenosine triphosphate
BHT	-	Butylated hydroxy toluene
CAD	-	Coronary artery disease
CD	-	Conjugated diene
CD36	-	Cluster of differentiation 36
CETP	-	Cholesterol ester transfer protein
COX	-	Cyclo oxigensase
CVD	-	Cardio vascular disease
DCFH-DA	-	2,7 Dichloro-dihydro fluorescin diacetate
DPI	-	Diphenylene iodonium chloride
EC	-	Endothelial cells
ECM	-	Extracellular matrix
EDTA	-	Ethylene diamine tetra acetic acid
EE	-	Electro elution
ELISA	-	Enzyme-linked immunosorbent assay
eNOS	-	Endothelial nitric oxide synthase
ERK1/2	-	Extracellular signal-regulated kinase1/2
FC	-	Free cholesterol
FFT	-	Fast fourier transform
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
INFγ	-	Interferon gamma
JNK	-	C-Jun N-terminal kinase
LCAT	-	Lecithin cholesterol acetyl transferase
LDL	-	Low-density lipoprotein
LDL-C	-	Low- density lipoprotein cholesterol
LDL-R	-	Low- density lipoprotein receptor

LOX	-	Lipoxygenase
Lp-PLA2	-	Lipoprotein associated phospholipase A2
LXR	-	Liver X receptor
M-CSF	-	Macrophage colony-stimulating factor
MAPK	-	Mitogen activated protein kinase
MCP-1	-	Monocyte chemoattractant protein -1
MetS	-	Metabolic syndrome
MMP	-	Matrix metalloproteinase
MPO	-	Myeloperoxidase
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-DIphenyl tetrazolium bromide
NAC	-	N-aetyl cysteine
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NADPH oxidase	-	Nicotinamide adenine dinucleotide phosphate
NBT	-	Nitroblue tetrazolium
NDGA	-	Nor dihydroguiateretic acid
NFKB	-	Nuclear factor kappa-B
NO	-	Nitric oxide
Nrf2	-	Nuclear factor erythroid- 2 related factor- 2
OxHDL	-	Oxidised-high density lipoprotein
p38 MAPK	-	p38 Mitogen activated protein kinase
PAF-AH	-	Platelet activating factor acetyl hydrolase
PAH	-	Polycyclic aromatic hydrocarbons
PARP	-	Poly(ADP- ribose) polymerase
PDB	-	Protein data bank
PEG	-	Poly ethylene glycol
PGI2	-	Prostaglandin I2
piHDL	-	pro inflammatory HDL
PKC	-	Protein kinase C
PL	-	Phospholipid
PLTP	-	Phospholipid transfer protein
PM	-	Particulate matter
PON	-	Paraoxonase
PPAR	-	Peroxisome proliferator-activated receptor
PS	-	Phosphatidyl serine
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
SSO	-	Sulfo succinimidyl oleate
TG	-	Triglyceride
TIMPs	-	Tissue inhibitor of metalloproteinases
TNFα	-	Tumor necrosis factor- alpha
TXA2	-	Thromboxane A2
UC	-	Ultra centrifugation
VCAM-1	-	Vascular cell adhesion molecule -1
XO	-	Xanthine oxidase

SYNOPSIS

India is undergoing a rapid health transition with rising burden of coronary artery disease(CAD) and its prevalence appears to be higher in South India with highest in Kerala. CAD is commonly due to obstruction of the coronary arteries by atheromatous-plaque that remains asymptomatic for decades. Overtime, atherosclerotic-plaque which consists of macrophages, cholesterol, fat, cellular waste products, calcium and fibrin, hardens and narrows the arteries, reduces the flow of blood to heart and can cause angina pectoris and myocardial infarction. It is well known that controlling the risk factors in individuals with CAD risk equivalents is effective in reducing the rates of CAD.

Atherosclerosis is a complex disease in which lesion development is the consequence of number of factors ranging from social, economic, physiological, lifestyle and biological (abnormal lipids, hypertension, diabetes). Yet an abundance of epidemiological evidence identifies low high density lipoprotein-cholesterol (HDL-C) as an independent risk factor for CAD. This relationship is supported by the potential anti-atherogenic properties of HDL, including cholesterol efflux from arterial macrophages, i.e. reverse cholesterol transport activity, antioxidative, antiinflammatory, antithrombotic, vasodilatory and antiapoptotic effects. However, many patients who experience a clinical event have normal, or even high levels of HDL-C. Recent data indicate that under some conditions, HDL can be modified, lose its cardio-protective effects and become dysfunctional and promote atherosclerotic-risk. It was hypothesized that functional loss in HDL with gain of pro-inflammatory properties could be a contributing factor for the excessive risk of CAD. However, the

underlying mechanisms responsible for generating dysfunctional HDL, the chemical and structural changes of HDL and the pro-atherogenic pathways exerted by the dysfunctional HDL remain largely unknown.

The present study was aimed at (i) identifying the prevalence of dysfunctional HDL in apparently healthy subjects and in patients having established CAD (ii) characterization of HDL together with underlying causative factors modulating HDL functionalities (iii) investigating the association between changes in HDL function and lipid profile, systemic oxidative stress, inflammation and HDL-associated biomarkers(iv) determining the effects of functional HDL and dysfunctional HDL on human macrophage functions relevant to atherosclerosis, specifically oxidative stress, inflammatory response, foam cell formation and delineating the associated molecular mechanisms.

In order to assess the functional alteration in HDL and other biochemical parameters, fasting blood samples (~10 ml) were collected from a total of 100 apparently healthy volunteers of both sexes, aged 25 to 55 years. Blood samples were also collected from clinically diagnosed CAD patients (n=40), who were admitted in Cardiology ward of this hospital. Patients with diabetes mellitus were excluded, as it is known to affect HDL functionality. Serum lipid profile, oxidative stress markers, and both the enzymatic and non-enzymatic antioxidants were quantitated using the standard assay methods. hsCRP and MMP-9 were measured based on ELISA and gelatin zymography respectively. The lipoproteins-HDL and LDL, were isolated from serum by sequential ultracentrifugation. HDL was then tested for its functionality in terms of its capacity to inhibit LDL oxidation using cell-free assay,

where DCFH- DA was used as a fluorescent probe. Generally HDL from healthy subjects shows remarkable capacity to inhibit LDL oxidation and is termed 'functional' HDL (nHDL). When the HDL fails to inhibit LDL oxidation, then it it is termed 'dysfunctional' HDL (proinflammatory/piHDL).

For cell culture experiments, human peripheral blood mononuclear cells were isolated from healthy volunteers and cultured under standard conditions for 24 h. These cells represent monocytes in an early stage of macrophage differentiation and are termed as monocytes-macrophages(Mo- MΦ).To differentiate monocytes into macrophages, cells were maintained in culture medium supplemented with 10% autologous human serum for 8 days.Cell viability was determined by trypan blue dye exclusion assay. Mo- MΦ or macrophages, maintained in culture were serum-starved overnight and then treated with medium containing PBS alone, nHDL or piHDL from CAD patients at varying concentration for different time intervals. Cells and culture medium were collected for further assays. Intracellular ROS formation was assessed based on ROS-mediated DCFH fluorescence. Oil red O staining and cholesterol measurement were used to detect cellular cholesterol accumulation qualitatively and quantitatively. TNF-ά, IL-10 and gelatinases in medium were determined using ELISA kits and gelatin zymography respectively. Western blot and RT-PCR were employed to study the expression levels of major proteins that are associated with foam cell formation. Hoeschst-PI staining, MTT assay, Comet assay and Flow cytometry were used to detect cell death mechanism.

The functional assay of HDL revealed that HDL from majority of healthy subjects exhibited remarkable antioxidant property as it significantly inhibited the

fluorescence signal generated by LDL oxidation. However, HDL from CAD patients did not possess antioxidant capacity to inhibit LDL oxidation and did not inhibit LDL oxidation. On the contrary, it further amplified LDL oxidation to a greater extent, demonstrating dysfunctionality in HDL and its pro-inflammatory nature. When the functional status of circulating HDL was assessed among healthy volunteers using larger sample size, it was observed that HDL functionality varied widely, irrespective of the level of HDL-C. Furthermore, HDL was found to be totally dysfunctional to inhibit LDL oxidation in 3% of healthy subjects during systemic inflammation (measured as high hsCRP & MMP-9) and oxidative stress. This finding indicates that all HDL are functionally not equivalent. Further characterization by gelatin zymography and western blot demonstrated for the first time, the association of matrix metalloproteinase-9(MMP-9) activity selectively in the dysfunctional HDL particle. Additional in vitro experiments confirmed that when MMP-9 was added to HDL, MMP-9 could form complex with HDL, and induce dysfunctionality in HDL, thereby providing evidence for a direct role of MMP-9 in HDL dysfunction. Since MMP-9 plays an important role in atherosclerotic-plaque formation and destabilization, the formation of HDL-MMP-9 complex may have important clinical implications. These results suggest that dysfunctional HDL can acquire pro-inflammatory property, due to its content of altered proteome, lipids, its oxidation products and possibly, due to its association with the protease-MMP-9. Elevated activities of serum MMP-9 was observed in CAD patients exhibiting dysfunctional HDL compared to healthy subjects. Apart from MMP-9(an intrinsic factor), environmental factors can modify HDL functionality. This study investigated whether particulate matter associated with air

pollution is capable of impairing HDL function. The results showed that exposure of HDL to PAH (polycyclic aromatic hydrocarbons-one of the most widespread organic pollutant, known for its adverse health effects), could negatively impact HDL functionality in a dose-dependent manner.

The next objective was focused on the pro-atherogenic effects of dysfunctional HDL in comparison to nHDL, using Mo- MΦ,the predominant cells involved in atherogenesis. Mo- MΦ after incubation with nHDL or piHDL (50μg/ml), were examined for pro-atherogenic properties. The results showed that piHDL treatment caused significant increase in ROS production, release of TNF-α and gelatinases-MMP-9 & MMP-2, compared to nHDL, indicating its pro-inflammatory property. In addition, the production of IL-10 (anti-inflammatory cytokine) was found to be decreased significantly in these cells when exposed to piHDL.

Macrophage foam cell formation in arterial wall is a fundamental, yet incompletely understood component in atherogenesis. The ability of HDL to promote cholesterol efflux from macrophages is an important anti-atherogenic mechanism. However, exposure of macrophages to piHDL , in contrast to nHDL, resulted in marked uptake of lipids from dysfunctional HDL, leading to formation of foam cell phenotype as noted by oil red O staining, with concomitant increase in total cholesterol content. These findings suggest that piHDL can impede macrophage-reverse cholesterol transport pathway, which inturn can increase the risk for atherosclerosis.

Investigating the mechanisms controlling the intracellular transport of lipid by dysfunctional HDL in comparison to nHDL in macrophages is essential in understanding foam cell formation. The possible involvement of certain receptors such as SRB1, CD36, and ABCG1 in mediating cholesterol influx/efflux in response to piHDL was investigated. Using western blotting and RT-PCR it was observed that piHDL profoundly upregulated the expression of CD36 in macrophages compared to nHDL, thereby facilitating cholesterol influx capacity of macrophages. In contrast, the expression levels of ABCG1 and SRB1 were suppressed by piHDL. The observed results provide clear evidence for the differential regulation of CD36 and SRB1 in macrophages by the two forms of HDL, that appears to be an important contributing factors for enhanced cholesterol uptake in macrophages and its reduced efflux. It was then identified that CD36 did not act alone, but it was activated in macrophages along with ERK/MAPK, in response to piHDL, which in turn led to lipid accumulation as well as pro-inflammatory response via activation of NFkB and subsequent release of pro-inflammatory markers- TNF-ά and MMP-9. Taken together, the results demonstrate that a novel CD36-ERK/MAPK-dependent mechanism is involved in macrophage lipid accumulation by piHDL, there by revealing the consequence of functional deficiency in HDL and its potential link to atherogenesis.

Lipid-laden foam cells are the characteristic pathological cells in atherosclerotic- plaques. One fate of foam cells that could interfere with removal of cholesterol from atherosclerotic lesions is foam cell death. Subsequent experiments were aimed at investigating the fate of macrophage- foam cells and elucidating the

mechanism that govern piHDL- induced cell toxicity. Exposure to piHDL was found to induce remarkable ROS production with concomitant decrease in antioxidants-glutathione, superoxide dismutase and catalase, as indicative of excess oxidative stress in macrophages. Further experiments demonstrated NADPH oxidase as the main enzymatic source of cellular ROS. Excess ROS formation as well as intracellular cholesterol accumulation was found to be toxic to cells as noted by significant cell death at 24 h. Additional experiments using, comet assay, flow cytometry and western blot revealed that macrophages exhibited DNA fragmentation, mitochondrial depolarization and cleavage of PARP (apoptotic marker protein), indicative of an apoptotic mechanism involved in cell death. These findings suggest that macrophage foam cells that do not efficiently function to remove cholesterol from lesion and under oxidative stress may ultimately undergo apoptotic cell death. Macrophage apoptosis is a prominent feature of atherosclerotic-lesions, and it is likely that dying macrophages without effective efferocytosis, influence lesion progression via release of lipid stores and potentially damaging degradative enzymes, such as MMP-9, into the extracellular space.

The present study demonstrates that HDL from CAD subjects, i.e.dysfunctional HDL, in contrast to HDL from healthy subjects, induces lipid uptake from dysfunctional HDL leading to foam cell formation in human macrophages. As a result the macrophage cholesterol efflux capacity of HDL in CAD patients is impaired. This study provides evidence that macrophage scavenger receptor CD36-ERK/MAPK signalling is required for piHDL-mediated proatherogenic responses, including foam cell formation and inflammatory property.

The data also demonstrate a significant role of piHDL in macrophage apoptosis that may further promote lesion progression. A remarkable finding in the present study is the previously unrecognized association of matrix metalloproteinase-9 with dysfunctional HDL and its pro-inflammatory property. Environmental pollutants can also influence HDL functionality as apparent from the observation that PAH converts functional HDL to dysfunctional HDL. All these results suggest a novel molecular link that can enhance the risk of atherosclerotic -cardiovascular disease in subjects with dysfunctional HDL. While there is a growing body of literature describing the cardioprotective effects of HDL, we still have much to learn about HDL, a heterogeneous particle.

1.1. INTRODUCTION

India is undergoing a rapid health transition with rising burden of coronary artery disease(CAD) and its prevalence appears to be higher in South India with highest in Kerala. More importantly, the disease catches young Indians, less than 40 years. Both men and women are prone to heart disease alike. CAD is commonly due to obstruction of the coronary arteries by atheromatous-plaque that remains asymptomatic for decades. Overtime, atherosclerotic-plaque which consists of macrophages, cholesterol, fat, cellular waste products, calcium and fibrin hardens and narrows the arteries, reduces the flow of blood to heart and can cause angina pectoris and myocardial infarction. Understanding the pathophysiology and improving the clinical management of CAD involves a knowledge of novel risk factors and biomarkers.

1.2. Pathogenesis of coronary heart disease

Atherosclerosis is by far the most frequent underlying cause of CAD. The intimal lesions are called atheroma or atheromatous plaques. According to the "response-to-injury hypothesis", atherosclerosis can be triggered by an injury to the endothelium (Ross, 1986) and injury can be triggered by any physical or chemical insults. When perturbation occurs, endothelial cells(ECs) secrete cytokines that trigger and maintain an inflammatory response resulting in recruitment and transendothelial migration of monocytes and T lymphocytes.The ECs also change shape, and increase the permeability to fluid, lipids, and leukocytes. The result is retention and oxidation of lipoproteins in the artery. Macrophages in the intima ingest the oxidized lipoprotein and they become foam cells. The continued

accumulation of foam cells in the intima lead to the formation of fatty streaks, an early objective sign of atherosclerosis(Ross, 1993). In addition, a number of cellular and molecular events including smooth muscle cell recruitment, proliferation and extracellular matrix protein synthesis also involves in atherogenesis(Amarjit Singh, 2012).

Several studies have shown that the risk of developing CAD is increased by smoking, hyperlipidemia, hypertension, diabetes, increased levels of C Reactive Protein(CRP), lipoprotein(a), hyperhomocysteinemia, metabolic syndrome, plasminogen activator inhibitor 1 and stressful lifestyle (Amarjit Singh, 2012). However, exercise and high density lipoprotein (HDL) and its major apolipoprotein, apoA-I, confer protection against atherosclerosis.

1.3. The role of HDL in atherosclerosis

HDL is recognised to have diverse antiatherogenic properties including promotion of reverse cholesterol transport (RCT), antioxidative, anti-inflammatory, antiapoptotic, antithrombotic, anti-infectious and vasodilatory effects (Eren et al., 2014). Several studies have identified low HDL-C(below 40 mg/dl) as an independent risk factor for coronary heart disease(Toth, 2004, Gotto, 2001). However, clinical events often manifest in patients with normal HDL-C levels in the range of 40–50 mg/dl (Ansell et al., 2003). Several large clinical trials have evaluated drug therapy on HDL-C and cardiovascular outcomes(Wright, 2013). All of the studies have demonstrated significant increases in HDL-C. But, pharmacological manipulation of HDL-C has not improved the cardiovascular

utcomes. This finding suggests that measuring HDL-C levels is not the most appropriate method for determining risk of CHD.

HDL is a heterogeneous particle and the potent antiatherogenic properties originate from its unique composition and structure (Eren et al., 2014). All HDL particles are functionally not equivalent. It can undergo pronounced compositional and functional modification in subjects under certain conditions associated with acute-phase response and inflammation. Several studies have shown that infection, inflammation, diabetes and CAD are associated with dysfunctional HDL(Hima Bindu et al., 2011). There is not enough clinical data to support this concept of functional deficiency in HDL as a contributing factor for the increased prevalence of CAD. The abnormality in HDL function raises the possibility of a pro-atherogenic effect of this particle. It was hypothesized that functional loss in HDL with gain of pro-inflammatory properties could be a contributing factor for the excessive risk of CAD. However the factors responsible for the generation of dysfunctional HDL and the pro-atherogenic pathways exerted by the dysfunctional HDL remain largely unknown. An understanding of the molecular mechanisms of HDL action may provide important insights into the development of new therapeutic advances targeted towards HDL for further reducing the atherogenic cardiovascular risk.

1.4. Significance of the study

Unlike other lipoproteins, physiological functions of HDL influence the cardiovascular system in favourable ways. The proper functional HDL particles are important for the reversal of atheroma formation in the artery. There is not enough clinical data to support this concept of functional deficiency in HDL as a contributing

factor for the increased prevalence of CAD. Therefore, it is highly essential to evaluate functional characteristics of HDL and macrophage influx/efflux function that better predict cardiovascular risk.

The thesis is an attempt to identify functional abnormality, in circulating HDL in healthy subjects and those with established CAD, understanding the mechanisms responsible for generating the dysfunctional HDL, and its pro-atherogenic effects on human macrophages, the key cell types involved in atherogenesis. The present study demonstrates that all HDL are not same in quality i.e. atheroprotective property. HDL from patients with established CAD, in contrast to HDL from healthy subjects, was found to be dysfunctional and pro-atherogenic as it promoted oxidative stress, inflammatory response and lipid accumulation in human macrophages thereby favouring formation of macrophage foam cells, an indication of atherogenesis. 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.

Objectives of the study

HDL has been shown to possess a wide variety of functional attributes which likely contribute to atheroprotection.This study mainly focused on the functional and compositional heterogeneity of HDL and its association with CAD to provide better understanding how HDL protects against atherogenesis.

The present study was aimed at

(i) Identifying the prevalence of dysfunctional HDL in apparently healthy subjects and in patients having established CAD

(ii) Characterization of HDL together with underlying causative factors modulating HDL functionalities

(iii) Investigating the association between changes in HDL function and lipid profile, systemic oxidative stress, inflammation and HDL-associated biomarkers

(iv) Determining the effects of functional HDL and dysfunctional HDL on human macrophage functions relevant to atherosclerosis, specifically oxidative stress, inflammatory response, foam cell formation and delineating the associated molecular mechanisms

REVIEW OF LITERATURE

2.1. Cardiovascular diseases

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and they include: coronary heart disease or coronary artery disease (CAD) such as angina and myocardial infarction or heart attack(disease of the blood vessels supplying the heart muscle), cerebrovascular disease e.g. stroke (disease of the blood vessels supplying the brain), peripheral arterial disease (disease of blood vessels supplying the arms and legs), rheumatic heart disease(damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria), congenital heart disease (malformations of heart structure existing at birth), deep vein thrombosis and pulmonary embolism (blood clots in the leg veins, which can dislodge and move to the heart and lungs)(Mendis etal, 2011)

Cardiovascular disease is globally considered as the leading cause of death with 80% of CVD related deaths being reported from low and middle income countries like India(Gupta et al., 2013). Coronary artery disease, also known as ischemic heart disease, is the leading killer of men and women worldwide. In 2004, coronary artery disease was responsible for 7.2 million deaths, or 12.2% of all deaths globally and has become a major burden upon public (http://www.who.int/).It is now affecting almost all sections of the society from young to old and most affluent to least affluent. Heart attacks and strokes are usually acute events and are mainly caused by a blockage that prevents blood from flowing to the heart or brain. The most common reason for this is a build-up of atherosclerotic plaques on the inner

walls of the blood vessels. But coronary disease can be due to other causes, such as coronary vasospasm, where the stenosis to be caused by spasm of the blood vessels of the heart. CADs are considered to be 'silent' diseases whose symptoms are not evident in a patient suffering from them till the disease is in an advanced state. Pharmacologic therapy, metallic stents, and coronary artery bypass grafts have been mainstays of treatment for ischemic heart disease.

2.2. Atherosclerosis and Coronary heart disease

Atherosclerosis is the principal cause of majority of coronary artery disease. The word atherosclerosis is derived from the Greek-meaning both softening (athere) and hardening (skleros)-and refers to a complex disease process affecting the major blood vessels of the body. In 1856, the German pathologist Rudolf Virchow, known as the "father of pathology," delineated the three physiological elements that interact to precipitate blood clotting (thrombosis) within the vascular system: the blood vessel wall, the components of the blood, and the flow characteristics of the blood(Virchow, 1856). He concluded from his research that atherosclerotic lesions were located within the intimal layer. The 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. In 1912, the American doctor James Herrick put the facts together and proposed that thrombotic occlusion of the coronary arteries plays a central role in myocardial infarction(Herrick, 1912). 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 (Hansson, 2005; Libby, 2002; Ross, 1993)

2.3. Coronary heart disease scenario in India

Coronary artery disease has been gaining importance as a major public health problem in India. India is undergoing a rapid health transition with rising burden of coronary heart disease(Srinath Reddy et al., 2005).Coronary artery disease is the foremost cause of disability and death the world over and is one of the top five causes of death in Indian population(Gupta et al., 2012).The global burden of diseases study reported the estimated mortality from CHD in India at 1.6 million in the year 2000(Murray and Lopez, 1997). Among adults over 20 years of age, there has been a two-fold rise in CAD in rural areas and a 6-fold rise in urban areas during the period from 1960 to 2002. Cross-sectional studies indicate the prevalence of CAD to be between 7-13% in urban and 2-7% in rural India(Gupta et al., 2008). Studies among Indian migrants in various parts of the world have documented an increased susceptibility to CAD in comparison to the native population (McKeigue et al., 1989). Epidemiological studies from various parts of India have reported the rising trends and a high burden in the levels of conventional risk factors such as diabetes, hypertension and metabolic syndrome which are largely determined by urbanization as evident from the urban-rural difference in the risk factors observed in India(Gupta, 2004; Mohan et al., 2007; Prabhakaran etal, 2007). Thus, in coming years, a phenomenal increase in the prevalence of CAD is expected in India, adding to the health burden due to CAD.

Lifestyle of populations in India have changed dramatically in the 20th century. A large section of the population has adopted an unhealthy lifestyle combined with decreasing physical activity, increasing stress levels and a higher intake of energy dense food and tobacco. As a consequence, CVD has emerged as the leading cause of death all over India, with CAD affecting Indians at least 5-6 years earlier than their western counterparts(Xavier et al., 2008). As a result, the Indian subcontinent suffers from a tremendous loss of productive working years due to CVD deaths. The huge burden of CVD in the Indian subcontinent is the consequence of the large population and the high prevalence of CVD risk factors. These estimates highlight the need for aggressive strategies for the prevention and control of CAD in India.

Kerala Scenario:

Kerala, with a population of over 34 million, is the most advanced state in epidemiological transition and has the highest prevalence of CAD risk factors in India(Thankappan et al., 2010). The prevalence of coronary artery diseases in Kerala is much higher than that in other states. CAD in Kerala is premature and malignant resulting in death at a very young age. Approximately 60% of CAD deaths in men and 40% of CAD deaths in women occur before the age of 65 years. The average age of a first heart attack decreased by at least 10 years in Kerala, in sharp contrast to a 20 year increase in many western countries(Soman et al., 2011; Thankappan et al., 2010). A community based study conducted in 1993 from the rural area of the southernmost district of Kerala reported a CAD prevalence of 7.4 %(Kutty et al., 1993). Another recent community-based cross sectional study(Krishnan et al., 2016)

revealed a high prevalence of CAD and coronary risk factors in Kerala. According to this study the prevalence of definite CAD in Kerala increased nearly three times since 1993 without any difference in urban and rural areas. Moreover, lifestyle diseases-CHD, diabetes, high blood pressure, and obesity, are paradoxically high and result in very high mortality and morbidity.

Women in Kerala are at very high risk of developing coronary artery disease because apart from all lifestyle factors, physical inactivity has cost them the biological protection of estrogen. Results from various studies indicate that not only the women in Kerala growing obese, they are also suffering from related complications like elevated lipids and blood sugar levels, hypertension which put them at high risk of coronary events(George et al., 2015; Krishnan et al., 2016). According to a study the prevalence of type 2 diabetes in women, in the less than 30 years age group, in Kerala is 3%(Thankappan et al., 2006). Polycystic ovarian syndrome and thyroid disease found among the adolescent girls as well as vitamin D deficiency can increase the rsik of CAD in women. The epidemic of CAD in Kerala warrants an urgent action in terms of high-risk interventions and other effective preventive strategies. Current scenario shows that CAD in Kerala has reached epidemic proportions and that urgent action is required in terms of high-risk interventions and other effective preventive strategies.

2.4. Risk factors for coronary heart disease

Coronary heart disease is a multifactorial disease with risk factors that tend to cluster and interact in an individual to determine the level of coronary risk. Atherosclerosis is a complex disease in which lesion development is the consequence

of number of factors ranging from social, economic, physiological, lifestyle and biological (abnormal lipids, hypertension, diabetes) factors. It is the result of interaction between genetic, lifestyle and environmental factors(saylos-Baixeras,2014). Extensive clinical and statistical studies have identified several factors that increase the risk of coronary heart disease(Gupta etal, 2008, Gupta ,2012; James,2013). The INTERHEART study(Yusuf et al., 2004), a large case–control study, performed in 52 countries of the world with 15152 cases of first myocardial infarction and 14820 controls, identified nine well-known coronary risk factors including abnormal lipids, smoking, hypertension, diabetes, a high waist-to-hip ratio, psychosocial factors, low fruit and vegetable consumption and lack of physical activity—as accounting for more than 90% cases of acute myocardial infarction world wide. An important finding of this study was a younger age of occurrence of acute myocardial infarction in South Asians. A cross-sectional study conducted among patients with established CAD have shown that in Keralites-irrespective of gender, diabetes and dyslipidemia are the major risk factor for CAD(James, 2013). In addition, other less well-known factors have received intense investigation over the past few years. These include both lipid particles [small, dense low-density lipoprotein (sdLDL), oxidized low-density lipoprotein, and apolipoprotein B] and nonlipid variables, such as metabolic factors (eg, impaired fasting glucose), thrombogenic/hemostatic factors (eg, fibrinogen), and inflammatory markers[high sensitivity C-reactive protein](hsCRP) (Fruchart J.C etal, 2004; Gupta, 2013).

The majority of CVD is caused by risk factors that can be controlled, treated or modified, such as high blood pressure, cholesterol, overweight/obesity, tobacco

use, lack of physical activity, diabetes, unhealthy diets and harmful use of alcohol. However, there are also some major CHD risk factors that cannot be controlled such as family history, ethnicity and age. Traditionally, risk factors for CHD have been categorized as behavioural, anthropometric and biochemical. Sometimes coronary artery disease develops without any classic risk factors. The possible factors include sleep apnea(repeated stop and start breething while sleeping), high sensitivity C-reactive protein, homocysteine[an amino acid] etc(Fruchart J.C etal, 2004; Gupta, 2013). Important risk factors for heart disease are listed in the figure 1.

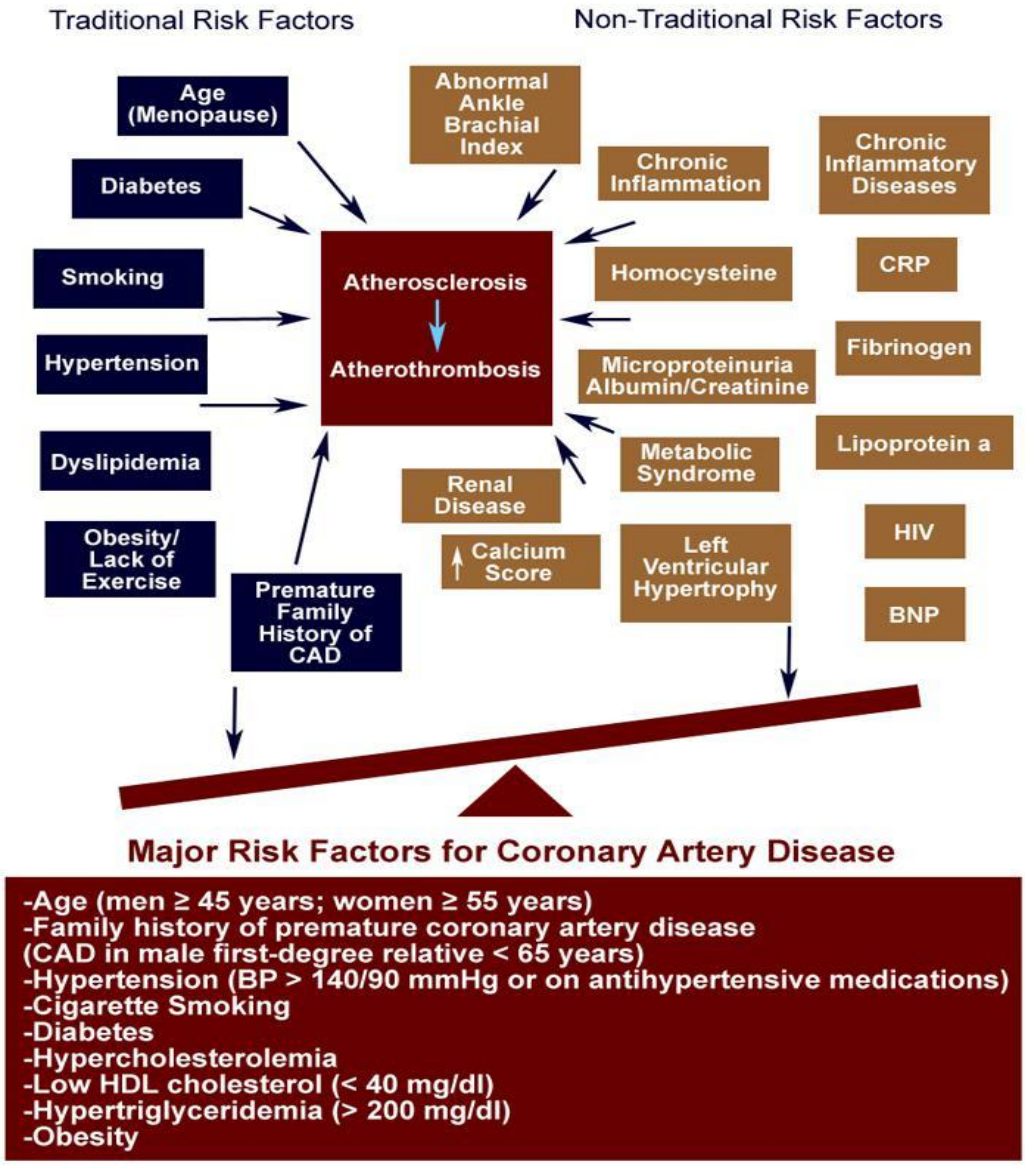


Figure 1: Traditional versus nontraditional risk factors for coronary artery disease. (*http://emedicine.medscape.com/article/164163*).

Atherogenic dyslipidemia has emerged as an important risk factor for CAD. Atherogenic dyslipidemia comprises a triad of increased blood concentrations of sdLDL, triglycerides and decreased HDL(Musunuru, 2010). Among factors that are associated with dyslipidemia, a low level of HDL-C(<40 mg/dl) is recognized as an independent risk factor for CHD(Gotto and Brinton, 2004). Moreover, low HDL-C is characteristic of atherogenic dyslipidemia and metabolic syndrome (MetS), which refers to the co-occurrence of several known cardiovascular risk factors, including insulin resistance, elevated fasting plasma glucose, abdominal (central) obesity, atherogenic dyslipidemia and hypertension. The presence of the MetS is believed to increase a patient's risk for CHD at any level of LDL-C (NCEP report, 2002). For effective treatment and prevention strategies to be put in place, the major risk factors associated with this disease must be identified.

Cumulative evidence from over two decades of clinical trials, have demonstrated the beneficial effects of LDL-C lowering agents-statins, or 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, in the reduction of CV event rates(Baigent etal,2010). However, several major statin trials have reported the existence of significant residual CVD risk in patients, despite increased reductions in LDL-C level, thus highlighting the need to focus beyond LDL-C levels(Sampson et al., 2012, Manjunath etal, 2013). The most notable lipid parameters implicated in residual CV risk are low HDL-C and high TG. This has led to heightened interest on HDL, as a potential target for therapy. It is well known that, in large populations, HDL-C levels are inversely related to the risk of atherosclerotic clinical events, however, in an individual, the predictive value of HDL-C level is far from perfect. It

is reported that, more than 40% of cardiac events occurred in subjects with normal HDL-C levels(Ansell et al., 2003; Castelli et al., 1986; Navab et al., 2005). 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). Consequent efforts at modulating HDL-C levels pharmacologically have failed to reduce residual CV risk (Barter PJ etal, 2007). Heterogenity of HDL function can contribute to CV risk independent of HDL-C(Khera et al., 2011). Further attention has turned to the quality, rather than the quantity, of HDL-C.The emerging notion that HDL particle quality has more predictive power than quantity has opened new areas of exploration.

2.5. Pathogenesis of atherosclerotic -CHD

Coronary artery atherosclerosis is the principal cause of coronary artery disease (CAD). Atherosclerosis in humans is a multi-factorial condition that develops over many years, and we are far from completely understanding its pathogenesis. Its progression is related to an interplay between environmental and genetic factors, with the latter exerting their effects either directly or via cardiovascular risk factors(Sayols-Baixeras et al., 2014). CAD can have various clinical manifestations, including stable angina, acute coronary syndrome and sudden cardiac death. Although clinical ischemic cardiovascular events usually appear after the fifth decade of life in men and the sixth decade of life in women, this process starts early in life, even during fetal development(Mecchia et al., 2009).

Marchand in 1904 introduced the term “atherosclerosis” describing the association of fatty degeneration and vessel stiffening(Aschoff, 1933). Briefly

atherosclerosis is a silent progressive chronic process characterized by accumulation of lipids, fibrous elements, inflammatory molecules in the walls of the arteries(sanz etal, 2012; Tabas,2013). This process is triggered by sometimes subtle physical or chemical insults to the endothelial cell(EC) layer of arteries. Injury to the endothelium , can be triggered by any number of insults, either alone or in combination. These include physical injury or stress as a result of direct trauma or hypertension ,turbulent blood flow at angulated or branch points, circulation of reactive oxygen species (free radicals), [e.g., from smoking or air pollutants], hyperlipidemia [high blood concentrations of LDL or very low density lipoprotein(VLDL)], chronically elevated blood glucose levels, homocysteinemia(Bonetti etal,2003). When perturbation occurs, endothelial cells secrete cytokines that trigger and maintain an inflammatory response(Pober J.S and Sessa.W.C, 2007). Circulating monocytes and T-lymphocytes are attracted to the sites of injury by chemoattractant cytokines (chemokines).The ECs begin to produce cell surface adhesion molecules such as VCAM-1, causing monocytes and T-lymphocytes to adhere to the endothelium and then migrate beneath it by squeezing between the endothelial cells. The ECs also change shape and the tight junctions between endothelial cells loosen, increasing the permeability to fluid, lipids and leukocytes. The result is retention and oxidative modification of LDL in the arterial wall .

Oxidized/modified LDL are potent chemotactic molecules that induce expression of vascular cell adhesion molecule and intercellular adhesion molecule at the endothelial surface, and promote monocyte adhesion and migration to the

subendothelial space. Monocytes differentiate to macrophages in the intima. The macrophages ingest oxidized-lipid, particularly oxidized-LDL, via scavenger receptors to become foam cells, and also have proinflammatory functions, including the release of cytokines such as interleukins and tumor necrosis factor(Glass and Witztum, 2001). Macrophages attempt to remove the oxidized LDL but are unable to degrade the cholesterol, which accumulates as droplets thereby giving a foamy appearance. Oxidative stress has therefore been recognized as the most significant contributor to atherosclerosis Macrophages retain the lipid they take up, and as they become more lipid-laden, they are referred to as "foam cells." The continued accumulation of foam cells in the intima lead to the formation of fatty streaks, an early objective sign of atherosclerosis(Ross, 1993; Stary et al., 1994).

It is now known that endothelial cells, leukocytes, and intimal smooth muscle cells play the key roles in the development of this disease. The cross-talk between monocytes, macrophages, foam cells, and T-cells results in cellular and humoral immune responses, and results in the production of several proinflammatory molecules(Libby, 2002; Witztum and Lichtman, 2014). This process continues with the migration of smooth muscle cells from the medial layer of the artery into the intima, resulting in the transition from a fatty streak to a more complex lesion. Smooth muscle cells produce extracellular matrix molecules, creating a fibrous cap that covers the original fatty streak. Foam cells inside the fibrous cap die and release lipids that accumulate in the extracellular space, forming a lipid-rich pool known as the necrotic core(Tabas, 2010). The accumulated cholesterol, cells and debris constitute an atheroma. Progressing atheroma often accumulates calcium. The

result of this process is formation of the second atherosclerotic lesion, the fibrous plaque. An atheromatous plaque is composed of cellular component in form of macrophages, smooth muscle cells, T cells, extracellular matrix including collagen, elastic fibres and proteoglycans and lipids(Kumar etal, 2010).

Two types of plaque can be defined depending on the balance between formation and degradation of this fibrous cap, ie, stable and unstable or vulnerable plaque. Stable plaques regress, remain static, or grow slowly over several decades until they may cause stenosis or occlusion(Osterud and Bjorklid, 2003). Unstable plaques are vulnerable to spontaneous erosion, fissure, or rupture, causing acute thrombosis, occlusion, and infarction, long before they cause hemodynamically significant stenosis. Stable plaques have an intact, thick fibrous cap composed of smooth muscle cells in a matrix rich in type I and III collagen(Finn etal, 2010). Protrusion of this type of plaque into the lumen of the artery produces flow-limiting stenosis, leading to tissue ischemia and usually stable angina. Vulnerable plaques have a thin fibrous cap made mostly of type I collagen and few or no smooth muscle cells, but abundant macrophages and proinflammatory and prothrombotic molecules. These plaques are prone to rupture. Intraplaque hemorrhage is also a potential contributor to progression of atherosclerosis(Doyle.B and Caplice.N, 2007). Plaque rupture involves secretion of metalloproteinases, cathepsins, and collagenases by activated macrophages in the plaque. These enzymes digest the fibrous cap, causing the cap to thin. Macrophages also stimulate thrombosis because they contain tissue factor, which promotes thrombin generation in vivo.

Plaque disruption[through superficial erosion or rupture of the fibrous cap] exposes the underlying thrombogenic core of lipid and necrotic material to circulating blood and its thrombogenic particulates, causing platelet adherence, aggregation, thrombosis, sudden occlusion of the artery lumen and usually an acute coronary syndrome(Libby et al., 2011; Sakakura et al., 2013). Endothelial apoptosis may be a critical step in the transition from a stable endothelialized plaque to plaque erosion and thrombosis(Durand et al., 2004). The most devastating consequences of atherosclerosis, such as heart attack and stroke, are caused by superimposed thrombosis. If the artery becomes blocked completely, then within a short period of time the heart becomes starved for oxygen (ischemia) and the heart muscle cells begin to die. This is medically termed a myocardial infarction(heart attack). The mechanism and the characteristics of the formation of the atheromatous plaque follow the main features of a chronic inflammation.

Clinically manifest atherosclerosis may be viewed as the culmination of four major steps. The steps include 1)initiation of endothelial activation and inflammation, 2) promotion of intimal lipoprotein deposition, retention, modification and foam cell formation, 3) progression of complex plaques by plaque growth, enlargement of the necrotic core, fibrosis, thrombosis, and remodeling and 4) precipitation of acute events. Due to the complexity of the lesions there can be no absolute differentiation between fatty streaks, fibrolipid lesions and complicated lesions. Inaddition not all lesions within a category are in the same stage of development in an individual, and all the 3 categories of lesions may co-exist in the same artery.

All stages of atherosclerosis—from initiation and growth to complication of the plaque—are considered an inflammatory response to injury mediated by specific cytokines (Hopkins, 2013)(figure 2).

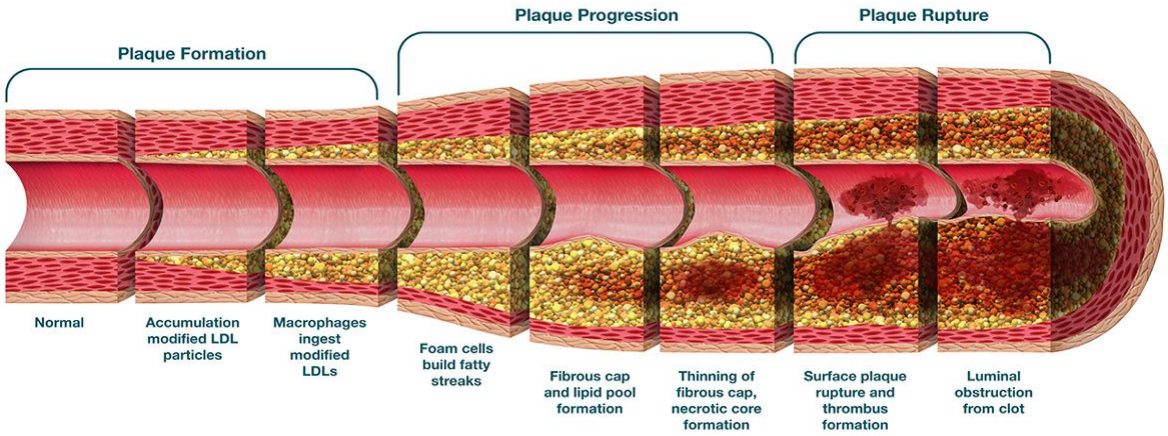


Figure 2: Atherosclerosis events occurring in an artery.cross sectional view).
[*http://chrisnierhaus.com*.]

2.6. Current theories of atherosclerosis

Atherosclerosis is a disease of large and medium-sized muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, and the build-up of lipids, calcium and cellular debris within the intima of the vessel wall. This build-up results in plaque formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow, and diminished oxygen supply to target organs. Exactly how atherosclerosis begins or what causes it is not known. But some theories have been proposed such as 'Insudation theory' or lipid infiltration theory, Encrustation theory, Inflammation and repair: a reaction to injury theory(Cullen P, 2005), The hemorheologic-hemodynamic theory(Gregory D. Sloop, 2016), Acidity theory of atherosclerosis (acidity evoked by stress, has an important role in

atherogenesis)(Monteiro, 2008), to explain the initiation and growth of atherosclerotic plaques. In the hemorheologic-hemodynamic theory, the name reflects the fact that the interaction of hemorheologic(blood flow) and hemodynamic (blood velocity, pulsatility, and arterial geometry) factors lead to atherosclerosis. The most widely accepted theory of atherosclerosis states that the process represents the body's attempt to heal in response to an endothelial injury.

2.6.1. The Response-To-Injury Hypothesis of Atherosclerosis

The response-to-injury hypothesis states that the initial event in the pathogenesis of atherosclerosis is injury to the endothelium(Ross, 1986). This theory suggests that even before development of the fatty streak, damage to the endothelium sets the stage for lesion development. Atherosclerosis occurs in the subendothelial space of medium-sized arteries at regions of disturbed blood flow and is triggered by an interplay between endothelial dysfunction and subendothelial lipoprotein retention (Tabas etal., 2015). Endothelial dysfunction compromises the integrity of the endothelial barrier to macromolecules, and leukocyte adhesion molecules on the surface endothelial cells are activated to promote the infiltration of macrophages into the subendothelium. The nature of the injury likely involves numerous factors and is different in different people depending on genetic and environmental conditions.

2.6.2. Endothelial dysfunction and atherosclerotic risk

The endothelium has emerged as the key regulator of vascular homeostasis. Alteration in endothelial function precedes the development of morphological atherosclerotic changes and can also contribute to lesion development

and later clinical complications(Deanfield et al., 2007). The endothelial lining of the vascular system comprises a dynamic interface with the blood and acts as an active signal transducer for circulating influences that modify the vessel wall phenotype. The vascular endothelium responds to these stimuli by synthesizing and metabolizing products that then act in an autocrine and paracrine manner to maintain vascular homeostasis(Tabas et al., 2007; Tabas etal., 2015). As an active, dynamic tissue, endothelium controls many important functions such as maintenance of blood circulation and fluidity as well as regulation of vascular tone, coagulation and inflammatory responses(Gonzalez and Selwyn, 2003). Physiologically, endothelial cells maintain a relaxed vascular tone and low levels of oxidative stress by releasing mediators such as endothelium-derived relaxing factor or nitric oxide (NO), prostacyclin (PGI2) and endothelin (ET-1), and controlling local angiotensin-II activit. In addition, the endothelium actively regulates vascular permeability to plasma constituents, platelet and leukocyte adhesion and aggregation and thrombosis. The arterial endothelium responds to flow and to shear forces in the blood via a pathway that leads to phosphorylation of endothelial nitric oxide synthase (eNOS), which in turn produces the potent vasodilator nitric oxide (NO), thus leading to vasodilatation(Eckardstein, 2006). In addition, the endothelium limits local thrombosis by producing tissue plasminogen activator, maintaining a negatively charged surface, and by secreting anticoagulant heparin and thrombomodulin. This state of balanced endothelial regulation of blood vessel function is, however, altered by a number of conditions resulting in endothelial dysfunction.

Endothelial activation represents a switch from a quiescent phenotype toward one that involves the host defense response. Endothelial dysfunction is characterized first by a reduction in the bioavailability of vasodilators, in particular NO, whereas endothelium-derived vasoconstrictors such as endothelin 1 are increased (Bonetti et al., 2003; Drexler, 1997). It is also characterized by a specific state of endothelial activation, which is characterized by a pro-inflammatory, proliferative and procoagulatory state that favours all stages of atherogenesis. Activation of endothelium results in expression of chemokines, cytokines, and adhesion molecules designed to interact with leukocytes and platelets and target inflammation to specific tissues to clear microorganisms(Hansson, 2005).This pathophysiological condition is associated with increased expression of adhesion molecules, such as VCAM-1, ICAM-1, E-Selectin and P-Selectin, increased synthesis of proinflammatory and prothrombotic factors, increased oxidative stress, and abnormal modulation of vascular tone, which may lead to different functional manifestations(Drexler, 1997). The fundamental change in this process is a switch in signalling from NO-mediated silencing and cellular processes towards activation of redox signalling. Dysfunctional endothelium promotes the adhesion of leukocytes to the arterial wall and their migration into the subintimal space, transformation of monocyte to macrophage,retention and oxidation of lipoproteins and also fails to inhibit the proliferation and migration of smooth muscle cells(Eckardstein, 2006). Macrophages ingest oxidized–lipids, which accumulate as droplets and giving a foamy appearance, foam cells. The acculumated cholesterol, cells and debris constitute an atheroma. In this regard, alterations of the endothelial phenotype into a dysfunctional state constitute a pathogenic risk factor for several vascular diseases

including atherosclerosis. 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(Amarjit Singh, 2012).

2.7. Inflammation and oxidative stress in atherosclerosis

2.7.1. Inflammation and atherosclerosis

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). Depending on the cause, inflammation can resolve rapidly or develop into a complex process involving different leukocytes as well as endothelial and mesenchymal cells. Multiple independent pathways of evidence now pinpoint inflammation as a key regulatory process that links multiple risk factors for atherosclerosis and its complications with altered arterial biology(Libby et al., 2009).

In atherosclerosis, the normal homeostatic functions of the endothelium are altered and promoting an inflammatory response. When the endothelial monolayer becomes injured or activated by various coronary risk factors, infections or physical stimuli, it expresses adhesion molecules that bind cognate ligands on leukocytes(Eriksson et al., 2001). Observations in human arterial specimens and many experimental models of atherosclerosis have identified monocyte recruitment as an early event in atherogenesis(Libby, 2002). Once the blood cells have attached, chemokines produced in the underlying intima stimulate them to migrate through the

interendothelial junctions and into the subendothelial space. Within the intima monocyte mature into macrophages under the influence of macrophage colony stimulating factor(M-CSF). Differentiation of monocytes into macrophages is critical for the development of atherosclerosis(Smith et al., 1995) and is associated with up-regulation of pattern-recognition receptors for innate immunity, including scavenger receptors and toll-like receptors(Libby et al., 2009). Once resident in the arterial wall, the blood-derived inflammatory cells participate in and perpetuate a local inflammatory response. M-CSF stimulation also increases macrophage expression of scavenger receptors for modified lipoproteins which engulf lipid. Ultimately, the cell is transformed into a foam cell, the prototypical cell in atherosclerosis.

Fatty streaks have focal increases in the content of lipoproteins within regions of the intima, where they associate with components of the extra cellular matrix(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; Skalen et al., 2002).

Oxidatively modified lipoprotein particles can induce a local inflammatory response(Miller et al., 2003). Platelets, when activated, can secrete preformed pro-inflammatory cytokines and shed a multipotent pro-inflammatory stimulus. 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.T lymphocytes encounter signals that cause them to elaborate inflammatory cytokines, such as interferon- γ [IFN-γ], interleukins[IL], or tumor

necrosis factor-α [TNF-α], which in turn can stimulate macrophages as well as vascular endothelial cells and SMCs(Marado, 2006). In parallel, macrophages proliferate and amplify the inflammatory response through the secretion of numerous growth factors and cytokines. A number of proinflammatory cytokines have been shown to participate in atherosclerotic plaque development, growth and rupture(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.In atherogenesis, NF-kB regulates the expression of cyclooxygenases, lipooxygenases, cytokines, chemokines and adhesion molecules (Józefa D bek, 2010). Inflammatory processes not only promote initiation and evolution of atheroma, but also contribute decisively to precipitating acute thrombotic complications of atheroma. Inflammatory mediators can inhibit collagen synthesis and evoke the expression of collagenases by foam cells within the intimal lesion. The activated macrophages 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. Cross-talk between T lymphocytes and macrophages heightens the expression of the potent procoagulant tissue factor. Thus, when the plaque ruptures, the tissue factor induced by the inflammatory signalling triggers the thrombus that causes most acute complications of atherosclerosis(Glass and Witztum, 2001; 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. It has been shown that NF-kB is an essential regulator of MMP gene expression, especially MMP-2 and MMP-9, which are critical in plaque rupture(Józefa D¹bek, 2010). Increased NF-kB activity was found in unstable regions of atherosclerotic plaques(Józefa D¹bek, 2010).

Considerable evidence suggests that impaired endogenous atheroprotective mechanisms occur at branch points in arteries, where the endothelial cells experience disturbed flow. For example, absence of normal laminar shear stress may reduce local production of endothelium-derived NO. This endogenous vasodilator molecule also has anti-inflammatory properties and can limit expression of VCAM-1(De Caterina et al., 1995). Augmented wall stresses may also promote the production of proteoglycans by arterial smooth muscle cells (SMCs) that can bind and retain lipoprotein particles, facilitating their oxidative modification and thus promoting an inflammatory response at sites of lesion formation. As this inflammatory process continues, the activated leukocytes and intrinsic arterial cells can release fibrogenic mediators, including a variety of peptide growth factors that can promote replication of SMCs and contribute to elaboration by these cells. As atherosclerotic lesions evolve, macrophage foam cells and smooth muscle cells can undergo apoptosis and contribute to lipid core formation and lesion complication. Some apoptotic cells may not disappear from the atherosclerotic lesions but accumulate in the fibrotic lesions due to impaired clearance or efferocytosis of apoptotic cells in plaques(Tabas, 2010).

Macrophages account for the vast majority of leukocytes found in atheromata. The cells of adaptive immunity, namely T lymphocytes and B

lymphocytes, also exist in atherosclerotic lesions, albeit in markedly lower numbers. Three fundamental biological processes generate atherosclerotic lesions: 1)LDL-cholesterol infiltration and activation within the intima of arteries, 2)endothelial activation and dysfunction, with adhesion molecule expression, 3)leukocyte recruitment from the blood stream with the consequent production of pro-inflammatory cytokines, chemokines and proteases, responsible for the maintaining of these inflammatory process within the vessel wall. These soluble mediators induce also the proliferation on smooth muscle cells and the production of acute phase reactants by the liver(Ridker et al., 1997).

Accumulating evidence suggests that inflammatory processes play a fundamental role in each of these stages in atherogenesis(Libby et al., 2011). Although the first event of atherogenetic cascade remains unknown, the inflammatory processes have to be considered as key components of the developing of the disease. Thus, a great challenge for future research would be the identification and development of promising novel anti-inflammatory therapies.

2.7.2. Role of Matrix metalloproteinases in atherosclerosis

Matrix metallo proteinases(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. MMPs are a very large family of calcium-dependent, zinc-containing endopeptidases produced by a variety of cell types, including endothelial, SMC, and monocytes(Bode et al., 1993; Woessner, 1991). 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). They are also involved in the biological activation of other proteins such as cytokines, growth factors and chemokines.

Approximately 20 different MMPs are identified. 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, and MMP-9) and the membrane-type MMPs (MT-MMPs)(Sternlicht and Werb, 2001). MMPs are secreted in a latent proform and require activation for proteolytic activity which 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. 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)(Klein and Bischoff, 2011). 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.

Gelatinase A[MMP-2] is ubiquitously expressed as a 72-kDa proenzyme [64-kDa active enzyme] and is capable of cleaving gelatine, 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. 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 (Thomas and Newby, 2010). 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.

There is growing evidence that MMPs are involved in all stages of the atherosclerosis process, from the initial lesion to plaque rupture. 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(Jeroen Bax, 2005). 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. Moreover, it was discovered that there was a correlation between increased MMP-9 expression and cap rupture, as well as a large lipid core(Heo et al., 2011). Another study determined that MMP-9 is associated with a risk of myocardial infarction(MI) and stroke(Jefferis et al., 2010). Immunocytochemistry, zymography, and in situ hybridization studies have demonstrated an increased expression of different MMPs in human atherosclerotic plaques. Furthermore, a significant increase in circulating MMP-9 levels was observed in patients undergoing carotid endarterectomy with evidence of ongoing spontaneous embolization(Loftus et al., 2000).

The messenger molecules that mediate communication between the various cell types involved in vascular proteolysis in atherosclerosis are poorly understood. However, there has been considerable interest in the role of the CD40/CD40L system. Ligation of CD40 on atheroma-associated cells causes activation of MMPs and enhanced production of cellular adhesion molecules and proinflammatory cytokines, chemokines and tissue factor and thus leads to progression of atherosclerosis, plaque destabilization, and the formation of intravascular thrombus(Ricketts, 2001). Homocysteine has been shown to activate MMPs via an increase in oxidative stress and acting as a signaling molecule on receptors like the peroxisome proliferator activated receptor-γ(PPAR-γ) and N-methyl-D-aspartate receptor. MMPs may also be activated by thrombin in atherosclerotic plaques. Several MMPs have been shown to be increased in cases of pathogenesis, including MMP-9, MMP-2, MMP-3, MMP-1, and MMP-8. However, MMP-10 has been sought as a potential alleviator of plaque, or a clot buster(Vacek et al., 2015). 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(Jeroen Bax, 2005). 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.

2.7.3. Oxidative stress and atherosclerosis

Oxidative stress and inflammation are intimately linked with both the evolution of cardiovascular disease and acute coronary syndromes. Oxidative stress

can be defined as an “imbalance between oxidants and antioxidant factors in favor of pro-oxidants and is central to the pathophysiology of atherosclerosis. Reactive oxygen species (ROS) are produced during normal aerobic processes in the body and are highly reactive with other biological molecules. The production of ROS is under tight control in healthy cells, but overproduction during metabolic dysfunction leads to cellular injury. An oxidative hypothesis of atherosclerosis was proposed in 1989 and it suggested modification of LDL as a primary reason of foam cell formation and development of atherosclerosis(Parthasarathy et al., 2010; Steinberg et al., 1989). It is well accepted now that oxidative processes and oxidized lipids play pivotal role in initiation and progression of the disease. An important initiating event for atherosclerosis may well be the transport of oxidized LDL(Ox-LDL) across the endothelium into the artery wall(Navab et al., 1996). Endothelial cells, smooth muscle cells (SMCs) and macrophages are the sources of oxidants for the oxidative modification of phospholipids(Antoniades et al.,2007). Hypercholesterolemia, diabetes mellitus, arterial hypertension, smoking and age increase the production of free ROS. The production of free oxidative radicals is believed to induce endothelial dysfunction, an initial step of atherogenesis. Oxidative stress leads to oxidation of LDL. The increased production of ROS reduces the production and consequently the bioavailability of NO, leading to vasoconstriction, platelet aggregation and adhesion of neutrophils to the endothelium. In fact, oxidative stress by hydrogen peroxide (H_2O_2) increases phosphorylation of tyrosin kinases, which leads to stronger binding of neutrophil cells on endothelium and alteration of vessel permeability(Vepa et al., 1999). Another mechanism through which oxidative stress by H_2O_2 affects atherogenesis is the production of transcription factors such as NF-κB and activator

protein 1 (AP-1), which participate in the expression of adhesion molecules such as vascular cellular adhesion molecules (VCAM- 1), intracellular adhesion molecules (ICAM-1), E-selectin and other cytokines(Bourcier et al., 1997). It seems that atherosclerosis is an inflammatory process strongly affected by oxidative stress.

In response to growth factors and cytokines and during normal metabolic events such as respiration and phagocytosis, eukaryotic cells produce oxidants. Although oxidation reactions are crucial for life, they can also be damaging. To compensate for this, the cells have evolved both enzymatic and nonenzymatic antioxidant mechanisms to protect against oxidant's toxic effects(Fridovich, 1978).The enzymatic mechanisms include the actions of enzymes such as superoxide dismutase(SOD), catalase, and glutathione peroxidase. The nonenzymatic antioxidants include glutathione, ascorbate, and α-tocopherol. However, in pathophysiologic circumstances, an excess of oxidants can overwhelm the scavenging capacity of cellular antioxidant systems. The subsequent oxidative stress damages the cell's lipids, membranes, proteins, and Deoxyribonucleic acid(DNA).

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 remodelling through collagen degradation, thus playing a central role in the development and progression of atherosclerosis and eventually in plaque rupture (Galkina and Ley, 2009). Growing evidence from investigations of animal models and correlative data from human studies implicate oxidative stress in the development of CVD. However, a better understanding of the ROS-dependent signal

transduction mechanisms, their localization, and the integration of both ROS-dependent transcriptional and signalling pathways in vascular pathophysiology is a prerequisite for effective pharmacological interventions for CVD.

2.8. Role of Lipoproteins in Atherogenesis

Human serum lipoproteins are soluble complexes of proteins (apolipoproteins) and lipids that represent the major cholesterol transport vehicles in both the intravascular and extravascular compartments. Lipoprotein particles are synthesized by the liver and intestine and mediate lipid transport from the intestine to the liver and between the liver and cells in the periphery of the body. Lipoproteins are macromolecular assemblies composed of lipids and proteins at variable ratios, densities and sizes. On the basis of their buoyant density lipoproteins are divided into 5 major classes: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL and HDL(Packard,1997). The plasma lipoproteins are the primary means of transport of cholesterol among tissues.

Apolipoproteins are part of a multi-gene family(Chan, 1989). ApoB100 is the principal protein constituent of LDL particles and by acting as a ligand for the LDL-receptor. It targets triacyl glycerol(TAG) and cholesterol for delivery to cells. Because of this activity, apoB100-containing lipoprotein particles are atherogenic. HDL mediates the efflux of cellular cholesterol, whereby excess cholesterol in cells in the periphery is transported to the liver and ultimately excreted from the body in the feces(Lund-Katz and Phillips, 2010). Apolipoprotein-mediated interactions of HDL particles with cell surface receptors and lipid transporters are critical for this process. HDL contains exchangeable apolipoproteins of the A, C and E families. In

particular, the apo B-containing lipoproteins (VLDL, IDL and LDL) are important for the delivery of cholesterol from the liver to peripheral tissues, while HDL appear to mediate the reverse process of movement of cholesterol from tissues back to the liver. Both of these transport processes are necessary for efficient whole body cholesterol homeostasis, because the liver is the major site of both the production and excretion of cholesterol. However, deviations from a proper balance of transport of cholesterol, either increases in LDL levels or decreases in HDL cholesterol flux, may result in accumulation of cholesterol in extrahepatic tissues(Babiak and Rudel, 1987). In most circumstances, it is assumed that an increase in plasma HDL-C concentration reflects an increase in the rate at which HDL is removing cholesterol from tissues and consequently a decrease in atherosclerosis. The importance of LDL-cholesterol in the development of atherosclerosis has long been recognized and LDL-C remains the primary target of therapy for the prevention of coronary heart disease. Nevertheless, increasing research attention over the past decade has been devoted to the heterogeneity of LDL particles and the atherogenicity of lipids and lipoproteins other than LDL. Particularly atherogenic forms of LDL include small, dense LDL particles and oxidized LDL. All lipoproteins that contain apolipoprotein B, such as LDL, VLDL, IDL and Lp(a) tend to promote atherosclerosis. However, these particles differ in their apolipoprotein and triglyceride content(Carmena et al., 2004). High levels of plasma triglycerides increase the risk of acute coronary events. Lipoprotein (a) [Lp(a)] plays a significant role in atherosclerosis and is one of the top five or six risk factors for cardiovascular disease(Morrisett, 2000).

2.8.1. Modified low-density lipoproteins and atherosclerosis

Abnormalities of lipid metabolism often lead to pathologic lipid accumulation in the vessel wall, oxidative and chronic inflammatory sequelae and the formation of atherosclerotic lesions, ultimately leading to clinical events. Oxidation of lipoproteins and in particular LDL, is a seminal event that mediates many pro-atherogenic and pro-inflammatory pathways. Many in vivo mechanisms exist to oxidize LDL, including transition metals such as divalent iron cations, heme, as well as a number of different enzyme systems, such as lipoxygenases, myeloperoxidase, NADPH oxidases, and nitric oxide synthases(Tsimikas and Miller, 2011). The oxidative modification hypothesis designates the oxidative change of LDLs as a crucial, if not mandatory, step in atherogenesis (Steinberg et al., 1989). During endothelial dysfunction, the endothelium expresses adhesion and chemotactic molecules and acquires an increased permeability to macromolecules. Hence, the entry of LDL particles in the arterial wall followed by their retention through the binding of apolipoprotein B100 to proteoglycans of the extracellular matrix is held to be a key-initiating factor in early atherogenesis(Tabas et al., 2007). The LDL particles trapped in the subintimal extracellular matrix are mildly oxidized by resident vascular cells(Navab et al., 1996). The oxidized LDL particles exert proatherogenic effects including stimulation of the resident vascular cells to produce monocyte chemotactic protein-1(MCP-1), granulocyte and macrophage colony-stimulating factors(Bae et al., 2009; Choi et al., 2009). These molecules promote monocytes recruitment and their differentiation into macrophages, which are able to further promote the oxidation of LDL through myeloperoxidase and ROS(Choi et al.,

2009; Parhami et al., 1993). The oxidized LDLs, are recognized by scavenger receptors on macrophages(Steinbrecher et al., 1984) and internalized to form foam cells, the hallmark of the atherosclerotic lesion. This generate a potent pro-inflammatory milieu. In addition, macrophages play a key role in atherogenesis through their proinflammatory action, which involves the production of interleukin-1β and TNF–α.

Besides contributing to the formation of lipid-laden macrophages, oxidized LDLs exhibit a wide array of biological properties, which are deemed to promote atherosclerosis(Giuseppe Maiolino, 2013). Oxidized LDLs(oxLDLs) exert chemotactic activity for monocytes and it is key for recruitment, activation and proliferation of monocytes/macrophages in the arterial wall. oxLDLs increase the expression of growth factors, such as platelet-derived growth factor and basic fibroblast growth factor by endothelial cells and macrophages, that stimulate migration and proliferation of SMCs and their production of collagen. oxLDL could also promote fibrous cap thinning by increasing secretion of MMP-1 and MMP- 9, thereby contributing to the occurrence of vulnerable plaques. Oxidized LDLs are cytotoxic to vascular cells and promote their apoptosis with ensuing release in the subendothelial space of lipids and lysosomal enzymes, enhancing the progression of the atherosclerotic plaque and the production of the necrotic core. Oxidized LDL stimulates platelet adhesion and aggregation, by decreasing endothelial production of NO and may contribute to vasoconstriction. In addition, they decrease the secretion of the tissue-type plasminogen activator and increase that of plasminogen activator

inhibitor-1 followed by a reduction of the fibrinolytic activity of endothelium(Maiolino G, 2013).

Foam cells could also be generated from macrophages internalizing native LDLs from the culture medium through micropinocytosis or by uptake of aggregated LDLs or LDL immune complexes(Kruth et al., 2005). Native LDLs are internalized by macrophages at a pace too low to account for foam cells formation owing to LDL receptor downregulation(Goldstein et al., 1979). Oxidative modification of LDLs increases their uptake by macrophages via scavenger receptors(Steinbrecher et al., 1984). Other main effectors in the development of atherosclerotic lesions are smooth muscle cells (SMCs), which are recruited from the tunica media to the subendothelial space, where they proliferate in response to mediators such as the platelet-derived growth factor. SMCs produce extracellular matrix molecules, including interstitial collagen and elastin, and build the fibrous cap that overlies the growing atherosclerotic plaque(Libby, 2008). Oxidation of LDLs induces immunogenic epitopes in their particles, which can be assessed by using murine monoclonal antibodies directed toward different oxidized LDLs epitopes and determining the immunogenic response to oxidized LDLs. Assays measuring oxidized phospholipids on apolipoprotein B-100 particles (OxPL/apoB) predict the presence and progression of femoral, carotid and coronary artery disease and predict new cardiovascular events independent of established risk factors(Tsimikas et al., 2006). This study suggests that pro-inflammatory oxidized phospholipids, present primarily on Lp(a), are significant predictors of the presence and extent of carotid and femoral atherosclerosis, development of new lesions and increased risk of

cardiovascular events. The oxidised phospholipid biomarkers may provide valuable insights into diagnosing and monitoring ardiovascular disease.

LDL is a major extracellular carrier of cholesterol and, as such, plays important physiologic roles in cellular function and regulation of metabolic pathways. The transport of cholesterol by modified LDL is diverted from its physiologic targets toward excessive cholesterol accumulation in macrophages and the formation of macrophage "foam" cells in the vascular wall. This pathologic deposition of modified lipoproteins and the attendant pro-inflammatory reactions in the artery wall lead to the development of atherosclerotic lesions(Miller et al., 2010). Continued accumulation of immunogenic modified lipoproteins and a pro-inflammatory milieu result in the progression of atherosclerotic lesions, which may obstruct the arterial lumen and/or eventually rupture and thrombose, causing myocardial infarction or stroke. The translation of experimental evidence in humans with studies aimed at the demonstration of the association of oxidative stress with cardiovascular events proved to be difficult and resulted in contrasting findings, particularly with administration of antioxidant therapy(Giuseppe Maiolino, 2013). Further understanding of the role of oxidation of lipoproteins may allow more rational targeted diagnostic and therapeutic modalities in clinical applications.

2.8.2. HDL and atherosclerosis

High density lipoproteins represent a heterogeneous population of lipoproteins in that they exist as functionally distinct particles possessing different sizes, protein content and lipid composition. The cholesterol required by peripheral tissues, including vascular cells, is provided both by new synthesis in the cells and by a

delivery from LDL. HDLs are the principal means by which excess cholesterol is removed from extrahepatic cells and transport this cholesterol back to the liver where it can be ultimately be excreted following conversion to bile acids(Small, 1988). HDLs oppose atherosclerosis directly, by removing cholesterol from foam cells, by inhibiting the oxidation of LDL and by limiting the inflammatory processes that underlie atherosclerosis(Besler etal,2012 ;Barter etal,2004). Thus, HDL-C interrupts the process of atherogenesis at several key stages. The complexity of these interactions renders HDL particles highly heterogeneous in their shape, size and surface charge. It is important to understand the complex and multifactorial ways in which HDLs protect the vasculature.

An abundance of epidemiological evidence identifies low HDL-C as an independent risk factor for CAD(Goldbourt ,1997; Assmann and Gotto, 2004; Lewington, 2007). Low levels of HDL-C [<40 mg/dl or 1mM/l] have clearly been shown to be an independent risk factor for premature heart disease, even at low levels of LDL-C. This relationship is supported by the potential anti-atherogenic properties of HDL(Besler et al., 2012). These finding that has led to the hypothesis that HDL protects from atherosclerosis(Vergeer et al., 2010). While reduction of LDL-C is the primary target of cardiovascular prevention, increasing attention has focused on HDL-C as a secondary target in order to address residual risk.

The antiatherogenic properties of HDL are thought to drive primarily from its role in RCT, but there is also evidence for antoixidant and antiinflammatory property. It is known that several genetic mutations can affect the structure, function and plasma concentration of HDL, but it is unclear how the mutations affect

cardiovascular risk. However, there is much that is unknown about HDL and an increasing need to understand, validate and quantify HDL's role in the atherosclerotic process. A clear understanding about the structure , functions and actions of the athero protective HDL may lead to a breakthrough in therapeutic applications of HDL in reducing health risk associated with CVD.

2.8.3. HDL structure, composition and metabolism

HDL is a class of heterogeneous lipoproteins containing approximately equal amounts of lipid and protein(Gordon and Rifkind, 1989). HDL particles are characterized by high density (>1.063 g/mL) and small size. 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(Phillips, 2013). The predominant species of HDL are spherical micro emulsion 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(Phillips, 2013). 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%). Cholesterol is the most characteristic component of the HDL lipidome as, in the form of HDL-cholesterol, 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. 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.

Apoprotein 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. In human plasma, about 25% of apoA-I is present in HDL particles, containing only apoA-I (LpA-I), the remaining HDL particles contain both apoA-I and apoA-II typically in a molar ratio of 1-2/1. 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. HDL also contains minor apoproteins such as Apo D, Apo M and enzymes such as paraoxonase (PON)-1, platelet-activating factor acetyl hydrolase(PAF-AH), and glutathione peroxidase-1,lipid transfer proteins such as lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP)(Hima Bindu et al., 2011). Recent experimental analytical techniques reveal that HDL particles actually contain over 50 different apoproteins. Unlike all the non-HDL lipoproteins[VLDL, IDL, lipoprotein(a) [Lp(a)], LDL], HDL particles do not contain apoB. Apo A1 is produced in the liver and intestine(Eggerman et al., 1991). Apo A1 is a critical component of nascent HDL and spherical HDL particle. The

structure of the discoidal HDL particles, which contain a segment of PL bilayer, are largely determined by the properties of the apoA-I molecule. Approximately 5% to 15% of apo A-I in human plasma is associated with particles with pre–β-electrophoretic mobility. These lipid-poor particles are increased in extravascular compartments where reverse cholesterol transport takes place(Assmann, 2004). The origin of HDL particles with pre–β-electrophoretic mobility is not entirely clear.

Knowledge of HDL structure at the molecular level is critical for understanding how this lipoprotein achieves the multiple functions. Apo A-I is the principal protein component of HDL and because of its conformational adaptability [amphipathic α-helix is the structural motif], it can stabilize all HDL subclasses. Human apoA-I is a 243 amino acid protein (molecular mass = 28.1 kDa)(Brouillette et al., 2001). The repeating amphipathic α-helical segments are critical for the ability of exchangeable apolipoproteins to interact with PL and stabilize HDL particles(Segrest et al., 1992). 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(Phillips, 2013).Hydropathy analysis of the amino acid sequence of apo A1 indicates that the C-terminal region of human apoA-I is very hydrophobic, consistent with this region having significant lipid binding ability(Saito et al., 2004b). ApoA-I is the only intact human apolipoprotein in the lipid-free state for which a high resolution structure is available to date. The crystal structure reveals an N-terminal anti-parallel four-helix bundle domain and a separate two-helix C-

terminal domain(Ajees et al., 2006) . It is difficult to obtain high resolution structures for HDL particles, because they are not suitable for study by X-ray crystallography and NMR. However, progress has been made by applying alternative methods to study homogeneous, reconstituted, HDL particles containing apoA-I as the sole protein. The generally accepted double-belt model for the structure of a discoidal HDL particle containing two apoA-I molecules. The two apoA-I molecules are aligned in an anti-parallel fashion so that the amphipathic α-helix spanning residues 121–142 in one apoA-I molecule is opposite the same helix in the other apoA-I molecule. Salt-bridges between the two apoA-I molecules help to stabilize this structure(Davidson and Thompson, 2007).

Remodeling of HDL Particles: Remodeling by plasma factors is a critical part of HDL metabolism and underlies the dynamic nature of HDL particles. Thus, the various subpopulations of HDL particles that exist in human plasma are continually interconverting due to the lipolytic and lipid transfer activities of the plasma factors(Lund-Katz et al., 2010). The changes in HDL particle shape and size caused by these plasma factors are also central to the participation of HDL in the RCT pathway. The key function of LCAT is to form CE while the other enzymes, hepatic lipase (HL) and endothelial lipase (EL), are involved in releasing fatty acids from PL and TAG. The lipid transfer proteins, CETP and phospholipid transfer protein (PLTP), are involved in the interconversion of HDL particles is frequently accompanied by the release of apoA-I molecules into the aqueous phase; rearrangements of HDL particles that are accompanied by a decrease in net surface area lead to desorption of apoA-I molecules and vice versa. Such cycling of apoA-I

molecules between HDL particles and the aqueous phase is critical for HDL metabolism. One of the key events in remodelling is the dissociation of lipid-free or lipid-poor apoA-I from spherical HDL by CETP, PLTP, and HL.The presence of apoA-II stabilizes the HDL particle and reduces its ability to be remodeled. Thus, apoA-II inhibits the ability of CETP to shrink spherical HDL particles and prevents apoA-I dissociation, the latter effect occurs because of stabilizing protein-protein interactions between apoA-I and apoA-II in the HDL particle surface. Progressive lipidation of apoA-I via this pathway generates discoidal HDL and recycles apoA-I back into the HDL fraction. This reduces the rate at which apoA-I is cleared from the circulation and helps to maintain circulating HDL levels (Lund-Katz and Phillips, 2010).

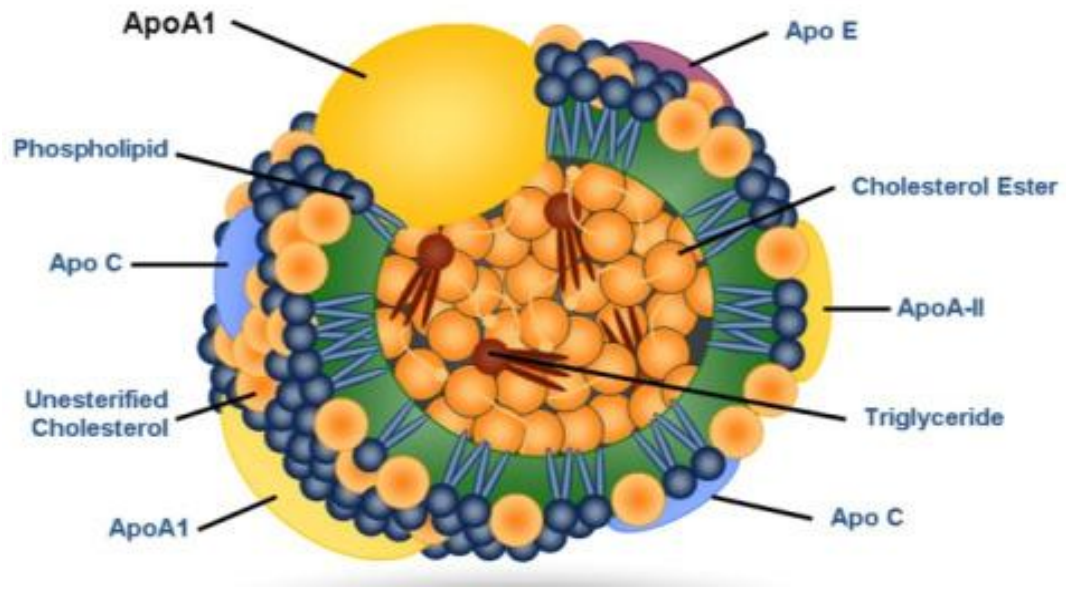


Figure 3: Structure of HDL. [*https://www.hdlforum.org*]

Origin of HDL: HDL originate as discoidal particles that are either secreted from the liver or assembled in the plasma from the individual constituents. Discoidal HDL consists of two or more apolipoprotein molecules complexed with phospholipids and unesterified cholesterol. Liver and intestine are both capable of synthesizing and secreting apolipoprotein (apo) A-I, the major HDL apolipoprotein that is required for normal HDL metabolism and are also primarily responsible for lipidating newly secreted lipid-poor apoA-I via ATP-binding cassette transporter A1(ABCA1)-mediated cholesterol efflux(Cuchel and Rader, 2006). ABCA1 is a ubiquitously expressed cellular lipid transport protein that promotes efflux of phospholipids and free cholesterol from cells to lipid-poor apoA-I, a form of "pre-β-HDL." and in its functional absence, apoA-I is rapidly catabolized.

HDL catabolism: In the RCT pathway, HDL-cholesterol is ultimately transported to and taken up by the liver. HDL-C undergo hepatic catabolism and excretion in bile.The most direct pathway is that of selective uptake of HDL-C by the hepatic HDL receptor SR-BI. SR-BI promotes "selective uptake," meaning that cholesterol is taken up, but that the HDL proteins, such as apoA-I, are not(Varban et al., 1998). Cholesterol from HDL can also be transferred to apoB-containing lipoproteins within the plasma compartment in exchange for triglyceride by CETP and ultimately returned to the liver via the LDL receptor pathway. HDL-C catabolism is mediated by 4 mechanisms: 1) hepatic uptake of larger HDL-C particles via hepatic scavenger receptor B1 (SR-B1) for excretion as bile, 2) metabolism of mature HDL-C by HL to smaller particles devoid of lipid and rich in Apo AI, 3) renal uptake of smaller HDL particles mediated by apo-E receptors such as cubulin [cubulin can bind Apo A1] 4)

LDL-R -mediated hepatic uptake of LDL-C and VLDL-C(Lewis, 2006; Moestrup and Kozyraki, 2000). The liver accounts for ~75% of total HDL cholesteryl ester turnover. In physiological terms, it is considered that the kidney cortex is a major site of catabolism for lipid-free and poorly lipidated apoA1. The final step in the reverse cholesterol transport pathway is excretion of cholesterol from the liver into bile, either directly or after conversion to bile salts and ultimately in the feces .

Complexity of HDL: The proteins associated with HDL can be classified in six major categories and include proteins involved in lipid, LP and HDL biogenesis and metabolism, acute phase proteins, protease inhibitors, complement regulatory proteins, and a few others[albumin, fibrinogen(Davidson et al., 2009). Furthermore the HDL proteome could be altered by pharmacological treatments and under conditions of infection, inflammation or tissue injury.Under this conditions acute phase response is triggered that causes huge alterations in hepatic protein synthesis in response to cytokines that alter HDL protein composition(Shah et al., 2013). In addition to proteins, a variety of lipids are also carried by HDL and some of them can be transformed to potent bioactive molecules. Furthermore, HDL carries and transports fat soluble vitamins, steroid hormones, carotenoids and numerous polar metabolites such as heneicosanoic acid, penitol and oxalic acid which were found to be significantly correlated with insulin resistance (Vickers and Remaley, 2014). HDL also transports small RNAs (Vickers et al., 2011). How all this proteins affects HDL metabolism and function remain to be studied.

2.9. Antiatherogenic functions of HDL

High-density lipoproteins represent a spectrum of particles that vary in their physicochemical and functional properties(Rosenson et al., 2011). There are several well-documented functions of HDL that may explain the ability of these lipoproteins to protect against atherosclerosis. The best recognized of these is the ability of HDL to promote the efflux of cholesterol from cells, such as macrophages in the artery wall, in the first step of the RCT pathway(Rye et al., 2009). This process may minimize the accumulation of foam cells in the artery wall. HDL also inhibit LDL oxidation, promote endothelial repair, improve endothelial function, have anti-thrombotic and anti-inflammatory properties and inhibit the binding of monocytes to the endothelium. In addition to preventing atherosclerotic lesion progression, HDL also promote lesion regression in animals(Rye, 2009). HDLs exert anti-inflammatory activity by inhibiting the expression of adhesion molecules by endothelial cells and the subsequent transmigration of monocytes. HDLs have antioxidant activity through the anti-oxidative properties of apoprotein A-I and the presence of enzymes such as paraoxonase, glutathione-peroxidase and PAF-AH. HDLs display an anti-thrombotic effect by inhibiting platelets aggregation, reducing von Willebrand factor levels, and enhancing the activity of activated protein C & S(Mineo et al., 2006). HDLs have a beneficial effect on endothelial function by activating endothelial NO synthase and enhancing NO release(Zulianii etal, 2007).

In this paradigm, HDL-C has been considered to be a marker of the potentially cardioprotective functions of HDL. However, several studies have suggested that the simple concentration of HDL-C may not always reflect HDL

function, with growing evidence that under some circumstances HDL function may be compromised despite high concentrations of HDL-C(Hutchins et al., 2014; Robert S. Rosenson, 2013).

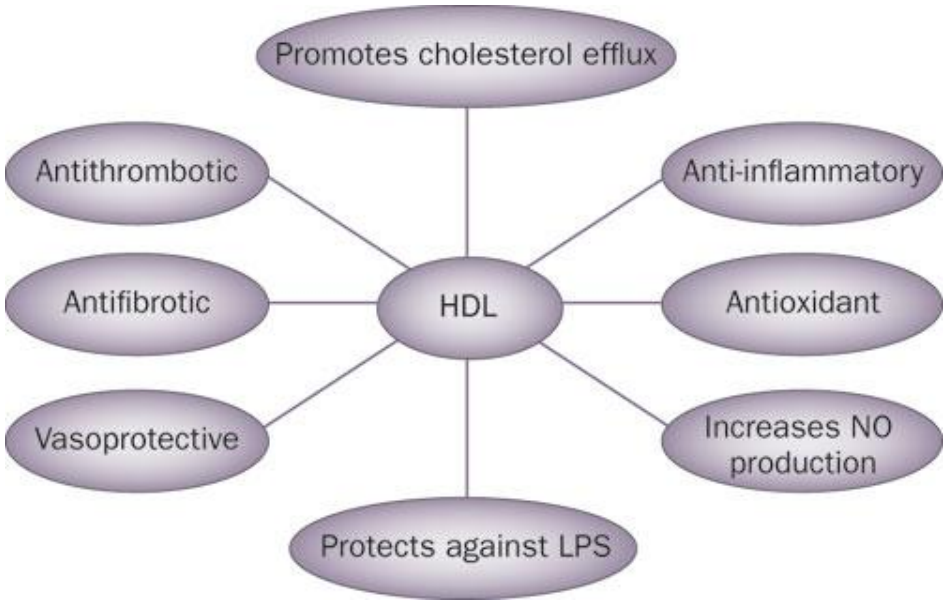


Figure 4: Anti atherogenic properties of HDL. [*www.nature.com*.]

2.9.1. HDL and Reverse Cholesterol Transport(RCT) pathway

The concept of "reverse cholesterol transport" was first introduced in 1968 by Glomset to describe the process by which extrahepatic (peripheral) cholesterol is returned to the liver for excretion in the bile and ultimately the feces(Glomset, 1968). Most types of cells in the body do not express the capability of catabolizing cholesterol, so cholesterol efflux is essential for homeostasis. Excess unesterified cholesterol (UC) is toxic to cells and therefore, cells have developed several ways to protect themselves against cholesterol toxicity. One key pathway is the efflux of

cholesterol to extracellular “acceptors”(ie. HDL). The return of this peripheral cholesterol to the liver is necessary to balance cholesterol intake and de novo synthesis and thus to maintain whole-body steady-state cholesterol metabolism(Cuchel and Rader, 2006).

The first step in reverse cholesterol transport is efflux of FC from the cell plasma membrane to HDL and, in the case of macrophages, four efflux pathways have been identified (Adorni et al., 2007). The two passive processes include simple diffusion via the aqueous phase and facilitated diffusion mediated by scavenger receptor class B, type 1 (SR-BI). Active pathways are mediated by the ATP-binding cassette (ABC) transporters-ABCA1 and -ABCG1, which are membrane lipid translocases. The combined deficiency of these transporters –ABCA1 and ABCG1, can lead to foam cell accumulation and accelerated atherosclerosis in mice(Yvan-Charvet et al., 2007). In the aqueous diffusion model, cholesterol molecules spontaneously desorb from cell membranes and are then incorporated into acceptor particles(HDL) after traversing the intervening aqueous space by diffusion. The net mass FC efflux from cells to HDL in the extracellular medium is promoted by metabolic trapping in which return of released FC to the cell is prevented by esterification when LCAT acts on HDL. The active process involves the interaction of phospholipid-depleted and cholesterol-deficient apolipoprotein (apo) A-I complexes with the ABCA1 in a process that results in the formation of a heterogeneous population of nascent HDL particles that are discoidal in shape and contain apoA-I, phospholipids, and free cholesterol. This HDL particles are excellent substrates for LCAT, the enzyme that generates most of the cholesteryl esters in

plasma. Cholesteryl esters are extremely hydrophobic and partition into the centre of the particles as they are formed. This converts discoidal HDL into the large spherical HDL particles that predominate in normal human plasma. Spherical HDL contain a core of neutral lipids (cholesteryl esters and some triglyceride) surrounded by a surface monolayer of phospholipids, unesterified cholesterol, and apolipoproteins(Phillips, 2013). LCAT generates a core of cholesteryl esters in a process that converts HDL particles from discoidal into spherical, α-migrating particles (small HDL). The interaction of spherical HDL particles with other active cellular transporters such as ABCG1 and passive diffusion of cellular cholesterol further increase the cholesterol load of HDL. However, it is often unappreciated that peripheral cholesterol efflux contributes <5% of the cholesterol content of HDL(Rosenson et al., 2012). In contrast to ABCA1, ABCG1 and SR-BI promotes macrophage efflux to mature HDL particles, which represent a much larger proportion of the HDL and apoA-I found in the plasma than the small pool of lipid-poor apoA-I(Cuchel and Rader, 2006; Kennedy et al., 2005).

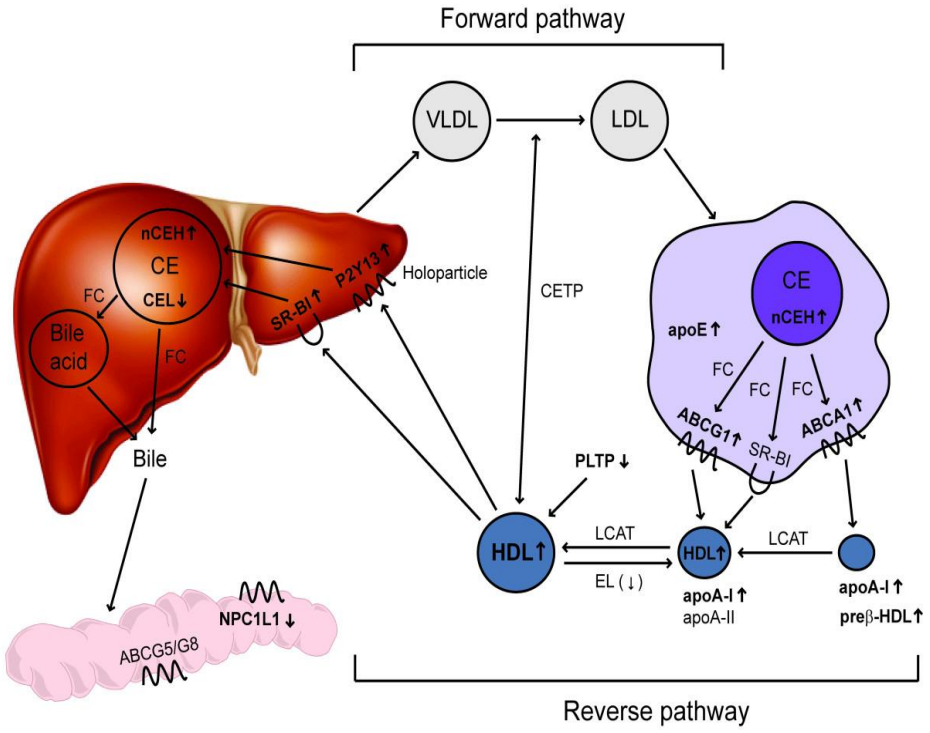


Figure 5: Reverse cholesterol transport pathway ***(****Annema etal, 2012).*

2.9.1.1. RCT and involvement of macrophage receptors

Removal of arterial cholesterol from macrophages via RCT is critical for preventing the development of atherosclerotic plaque(Cuchel and Rader, 2006). Studies on macrophage RCT in mice have demonstrated that apo A I and ABCA1 play essential roles in promoting RCT and they are considered as anti atherogenic proteins(Wang et al., 2007; Zhang et al., 2003). These two proteins interact to mediate the first step in RCT, the efflux of cellular cholesterol. ABCA1 is a full transporter whose expression is up-regulated by cholesterol loading, which leads to enhanced FC efflux. A two-state mechanism may explains the active transport activity of ABCA1. Binding and hydrolysis of ATP by the two cytoplasmic

nucleotide-binding domains control the conformation of the transmembrane domains so that the extrusion pocket is available to translocate substrate from the cytoplasmic leaflet to the exofacial leaflet of the bilayer membrane. ABCA1 actively transports phosphatidylcholine, phosphatidylserine, and sphingomyelin with a preference for phosphatidylcholine(Quazi and Molday, 2013). Inaddition to ABCA1, another transporter ABCG1 and SRB-1, can promote FC efflux to mature HDL particles(Adorni et al., 2007).SRB1 is a HDL- recptor that mediates selective cholesterol ester(CE) uptake into cells that involves movement of CE down its concentration gradient from HDL to cells. In addition to promoting delivery of HDL-CE to cells, SRB-1 also enhances efflux of cellular cholesterol to HDL(Gu etal, 2000). HDL binds to SR-BI with high affinity and this interaction promotes cholesterol efflux to the docked HDL particles. The facilitated movement of cholesterol molecules between the PL bilayer of the plasma membrane and the HDL particle bound in the appropriate conformation occurs by diffusion through a nonpolar channel (tunnel) formed by the extracellular domain of the SR-BI molecule. Because the concentration of free cholesterol is higher in the plasma membrane than in the HDL particle, efflux of free cholesterol is favored. ABCG1 mediates cholesterol transport to HDL through its ability to translocate cholesterol and oxysterols across membranes. ABCG1 is located in endosomes, where it promotes transport of FC from the endoplasmic reticulum to the plasma membrane. Expression of ABCG1 enhances FC and PL efflux to HDL but not to lipid-free apoA-I(Phillips ,2013). Expression of ABCG1 increases both the cell FC pool available for efflux and the rate constant for efflux.

2.9.2. Antioxidant and anti-inflammatory properties of HDL

The specific physiologic role of HDL in protection from atherosclerosis is multifactorial. HDL may be regarded as a naturally occurring antioxidant that plays a role in vascular protection independent of RCT(Negre-Salvayre et al., 2006). The mechanism by which HDL performs antioxidant activity is complex and multifactorial. The antioxidant activity of HDL is primarily mediated via the inhibition of the oxidation of LDL(Navab et al., 2004) with a subsequent reduction of the cellular lipid uptake by the monocyte macrophage system(Nicholls et al., 2005). The uptake of oxidized LDL within the smooth muscle and macrophages is unregulated and results in progressive accumulation of lipids within the vascular system. Transition metals have played a role in oxidative stress and HDL has been demonstrated to exhibit chelation properties due to be presence of proteins such as ceruloplasmin on the surface of the lipoprotein, although the clinical relevance is controversial. Lipid peroxidation products are cytotoxic and predispose to atherosclerosis. HDL has been demonstrated to accept hydroperoxides from oxidized membranes in invitro studies, which would potentially provide a pathway for excretion or detoxification. Additionally, the apoprotein (Apo A-I and Apo A-II) constituents of HDL may play at least a partial role in the antioxidant activity. Apo A-I has been demonstrated to reduce lipid hydroperoxides into redox inactive compounds, which thus terminates the chain reactions of lipid peroxidation (Mohinder Bansal, 2014). Other apolipoproteins associated with HDL may act along side apoAI to inhibit lipid hydroperoxide accumulation. Apolipoprotein E (apoE) appears to display this anti-oxidative activity, while apolipoprotein M has recently

been reported to display anti-oxidative functionality in transgenic mice in addition to facilitating PON1 activity(Soran et al., 2015). The HDL particle is also associated with a variety of enzymes,PON-1, PAF acetylhydrolase, glutathione peroxidise-1, that demonstrate antioxidant activity and are involved in the functionality of HDL(Tomas et al., 2004; Karlsson et al., 2015). Also phospho lipase A2(PLA2) on HDL is likely to contribute anti-oxidative activity by the same mechanism as PON1, by hydrolyzing lipid hydroperoxides (Soran et al., 2015). Variants of Apo A1 such as Apo A1 Milano provide support for the role of HDL as an antioxidant. The recognition of Apo A1 Milano has provided considerable insight into the functionality of the HDL particle independent of circulating levels. Apo A1 Milano has been demonstrated to exhibit a variety of physiologic functions that may play an additive role in protection from atherosclerosis independent of the classic view of reverse cholesterol transport(Alexander et al., 2009). Further HDL protects against the induction of endothelial dysfunction and demonstrates both antioxidant and antiinflammatory effects(Barter et al., 2004).

HDL particles display multiple anti-inflammatory actions which collectively may contribute to suppression of a chronic inflammatory response in the arterial wall (Sorci-Thomas and Thomas, 2012). One pathway that seems to be important in atherosclerosis and autoimmunity is its role in modulation of T cell activation. Cholesterol efflux to HDL has a significant role in suppressing inflammation. HDL and apoA-I suppress inflammation by reducing the lipid-raft environment by promoting FC efflux from tissues and inflammatory cells, such as T cells and macrophages, resulting in an increased fraction of regulatory T cells that attenuate

inflammation(Sorci-Thomas and Thomas, 2012). The multiple effects of HDL on the immune system suggest that several mechanisms of action may be operative. HDL also possesses antiinflammatory properties by virtue of their ability to inhibit the expression of adhesion molecules in endothelial cells, thus HDL reduce the recruitment of blood monocytes into the artery wall. At the molecular level an interference with classical proinflammatory transcription factors such as the nuclear factor kappa B (NF-κB) has been observed (Barter, 2004). HDL has been proposed to inhibit NF-κB activation by interrupting a sphingosine kinase signalling pathway upstream of NF-κB. Distinct anti-inflammatory effects of the two main HDL receptors SR-BI and ABCA1 have been described in macrophages(Yvan-Charvet et al., 2010). Antioxidative properties of HDL are closely linked to its anti-inflammatory potential.

2.9.3. Antiapoptotic property of HDL

Apoptotic cell death following injury of vascular endothelium is assumed to play an important role in the pathogenesis of atherosclerosis(Nofer, 2001). HDL particles display potent cytoprotective actions. HDL protects both macrophages and endothelial cells from apoptosis induced by loading with free cholesterol or by oxidized LDL or by chylomicron remnants, TNF-alpha, proteins of the complement system, and growth factor withdrawal. Major intracellular mechanisms underlying the anti-apoptotic actions of HDL include preservation of mitochondrial integrity, abrogation of caspase cascade activation, reduced fragmentation of nuclear DNA, blockage of cytotoxic calcium signalling pathways, and activation of the survival Akt pathway(Kontush,2014). HDL receptor, ABCG1, is critical to the anti-apoptotic

effects of HDL in macrophages and endothelial cells. Cytoprotection is also related to intracellular antioxidative actions of HDL(Terasaka et al., 2007).

2.10. HDL-raising therapies

Low HDL-C levels often reflect a genetic abnormality, although they can also be pushed downward by a high blood level of TGs or by cigarette smoking, inactivity, or hypertension, as well as by a diet very high in carbohydrates or polyunsaturated fats(Chopra,2015). Approaches to raise HDL-C levels and subsequently promote RCT include lifestyle modifications, [exercise and weight loss; smoking cessation; dietary modifications with consumption of foods high in n-3 polyunsaturated fats], standard pharmacologic therapy [includes niacin, fibrates, and statins] and several emerging therapeutics based on metabolic targets involved in RCT(Cimmino et al., 2015; Kapur et al., 2008) .

Emerging therapeutics: HDL-C delipidation therapy(utilizes plasmapheresis), 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(Kapur et al., 2008). HDL infusion therapy using apo A-I Milano (recombinant apo A-1 Milano/phospholipid complexes) offer a potential therapy to modulate the clinical sequelae that result from rupture of unstable atherosclerotic plaques(Hegele, 2004).

A considerable body of evidence supports the correlation between HDL-C levels and cardiovascular risk. 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. As a result, there is not enough data to support guidelines recommending aggressive increase in HDL-C levels. Given the complexity of HDL metabolism, serum levels of HDL may not be an adequate indicator of efficacy. The major challenge is to develop laboratory assays that quantify the various HDL functions that may improve CVD risk assessment and augment the evaluation of HDL-modifying therapies.

2.11. Functionally modified HDL and atherosclerosis

The past decade has seen a paradigm shift in our understanding of HDL and its relationship to atherosclerosis. HDL is most widely recognized for its ability to shuttle cholesterol from the periphery to the liver for catabolism/excretion during the process of RCT. It is well known that, in large populations, HDL-C levels are inversely related to the risk of atherosclerotic clinical events; however, in an individual, the predictive value of an HDL-cholesterol level is far from perfect. Many patients who experience a clinical event have normal or even high levels of HDL-C(Baron and Baron, 2007; Hima Bindu et al., 2011).Raising HDL levels in animal models by infusion or over expression of apolipoprotein A-I has shown clear vascular improvements, such as delayed atherosclerotic lesion progression and accelerated lesion regression, along with increased RCT(Fisher et al., 2012). However, human studies have cast some doubt on the HDL hypothesis. A genetic method called mendelian randomization found that a score derived from 14 common genetic variants that is associated with HDL-C levels, and not other lipoprotein traits, is not associated with myocardial infarction(Voight et al., 2012). Furthermore, two drug trials, one of the CETP inhibitor torcetrapib, and the

other of extended-release niacin, did not show beneficial cardiovascular outcomes despite increased HDL-C levels(Barter etal, 2007). This may be due to functional aleteration in the HDL particles. Ideally, a means of monitoring HDL function in the circulation should reflect what is occurring within the diseased artery wall. Recent attention has turned to the quality, rather than the quantity, of HDL. There are several well documented functions of HDL that may explain tha ability of HDL to protect against atherosclerosis(Besler etal, 2012; Navab etal, 2011; Hima Bindu etal, 2011; Mineo et al., 2006). If HDL particles perform these biologic tasks they are termed, "functional HDL"(Smith, 2010). However, all HDL are functionally not equivalent. It can undergo pronounced compositional and functional modifications. Under conditions of infection, inflammation, or tissue injury, the acute-phase response(APR) is triggered, causing huge alterations in liver protein synthesis patterns that translate to remarkable changes in HDL protein composition(Shah, 2013). In addition to proteins consistent with traditionally accepted roles in lipid transport, HDL carries surprising constituents, such as members of the complement pathway, protease inhibitors involved in hemostasis, acute-phase response proteins, immune function mediators, and even metal-binding proteins(Shah et al., 2013). Negative APR proteins include apoA-I, transthyretin, and retinol-binding protein.These proteins tend to be replaced in HDL by positive APR proteins, which include SAA, apoJ/clustrin, and lipopolysaccharide-binding protein (LBP). The positive and negative APR proteins residing in HDL appear to coordinate short-term inflammatory and repair processes. Thus when anti-oxidant and anti-inflammatory functions of HDL are overwhelmed by pathological processes, such as inflammation, HDL is converted into a 'dysfunctional' pro-inflammatory particle(Smith, 2010a).

Plasma concentrations of HDL-C are insufficient to capture the functional variation in HDL particles and the risk of CVD associated with HDL. HDL-C content is the one minor HDL component that exist in a deciliter of plasma. So, HDL-C levels has no major relationship with how the HDL-particle concentrations are being dynamically remodeled or the state of HDL functionality(Shah, 2011).

HDL function can be measured by several in vitro assays. Several studies have clearly shown that specific functional readouts, such as capacity for cholesterol efflux or anti-inflammatory index , may be better indicators than the HDL-C number for predicting CAD risk in an individual(Ansell et al., 2003; Khera et al., 2011). Several animal models associated with dysfunctional HDL were established. Using SR-BI-null mice, numerous studies(Gong et al., 2003; Van Eck et al., 2007) 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. Several other studies have shown that infection, inflammation, diabetes, and coronary artery disease are associated with dysfunctional HDL (Hima Bindu et al., 2011 Lewington et al., 2007; Smith, 2010a, Distelmaier et al., 2015). South Asian immigrants with a high incidence of metabolic syndrome and diabetic complications also display HDL with inflammatory properties(Shah and Kanaya, 2014). Antioxidant capacities of HDL were reported to be significantly impaired in the acute phase of acute myocardial infarction as well as in the chronic stable phase 1 year after the event(Distelmaier et al., 2015). 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.

The potent atheroprotective properties of HDL particles originate from their unique composition and structure. HDL particles are quite heterogeneous, encompassing a range of sizes, densities and contain multiple surface apolipoproteins, varying concentrations of cholesterol and phospholipids, and various antioxidant or pro-oxidant enzymes, which are now recognized as significant factors in the balance of pro- and antiatherogenic metabolism(Eren et al., 2012a). Even though there are several evidences showing the prevalence of dysfunctional HDL in patients with acute coronary syndromes, the underlying pathways through which HDL might get converted into a proinflammatory stage are quite unknown.

HDL are susceptible to structural modifications mediated by various mechanisms including non-enzymatic oxidation, glycation, homocysteinylation or enzymatic modification/ degradation(Ferretti et al., 2006). Recent studies from our laboratory have demonstrated that in vitro oxidative modification of HDL loses its atheroprotective functions and exerts proinflammatory response and oxidative stress in human monocytes/macrophages (Soumyarani and Jayakumari, 2012). Furthermore, Oxidized HDL induces cytotoxic effects in monocytes (Soumyarani and Jayakumari, 2014). Evidence for the presence of modified HDL[cross-linked apoproteins] in atherosclerotic tissues has also been reported(Nakajima et al., 2000). Recent studies indicate HDL and their major structural protein, apolipoprotein A1 (apoA1), recovered from human atheroma, are dysfunctional and extensively

oxidized by myeloperoxidase (MPO)(Huang et al., 2014). This study identified the structural basis of an abundant dysfunctional form of apoA1/HDL within the artery wall – MPO-catalyzed site-specific oxidation of Trp72 of apoA1 forming an oxindolyl alanine (2-OH-Trp) moiety. Thus, within the artery wall, MPO-dependent oxidative modification of apoA1 appears to be highly correlated with the atherosclerotic disease process. As a result, HDL may lose its normal function and acquire pathological functions. Hence HDL may be viewed as a shuttle that can be anti-inflammatory or proinflammatory, depending on its cargo of proteins, enzymes, and lipids. Since HDL represents a heterogeneous molecule any alteration occurring in its composition and or structure may impair its biological function and convert it into a pro-inflammatory molecule. The attenuated atheroprotective properties of HDL in metabolic disease raise the possibility of an indirect putative proatherogenic effect of these particles. However, the precise mechanisms by which HDL may impact cardiovascular health and disease are complex and remain to be fully understood. An understanding of the molecular mechanisms of HDL action may provide important insights into the development of new therapeutic advances targeted towards HDL for further reducing the atherogenic cardiovascular risk.

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, PD98059, 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, Dimethyl sulphoxide, Gentamycine, Streptomycine, Sodium citrate, Sucrose, Penicillin, Calcium chloride, Sodium bicarbonate, Paraformaldyhyde, Riboflavin, Folin‘s reagent, Glycerol, PEG8000, DNAse, Thiobarbituric acid, Guanidine Hydrochloride, Agarose, Filter membranes, Protease inhibitor cocktail, Color burst electrophoresis marker, Thiazolyl Blue Tetrazolium Blue(MTT), NADPH, glutathione reduced, allopurinol, Nordihydroguaiaretic acid(NDGA), Diphenyleneiodonium chloride(DPI), Indomethacin, Sodium azide , Methionine, Riboflavin, Potassium cyanide , Hydrogen peroxide, FITC-annexin V apoptosis detection kit, PVDF membranes etc. were purchased from Sigma (St. Louis, USA). Syringe filter was purchased from Millipore. Tissue culture plates were

purchased from Nunc, Denamrk. PCR primers were synthesized and purchased from CalBiochem. MMLV –reverse transcriptase, RNasin, dNTPs, Taq 72 DNA polymerase, Trizol reagent, CD36 primer were purchased from Promega Corporation, USA. Polypropylene tubes for culture were purchased from BD Bioscience. Reagent kits for Cholesterol and Triglycerides were purchased from ASPEN lab, Delhi and Phospholipid kit from FAR diagnostics, Verona, Italy. hsCRP reagent kit was obtained from hyphen Biomed, France. Super signal West Femto Substrate kit for chemiluminiscent assay , TNF-alpha and IL-10 kit were purchased from Thermo scientific, Rockford, USA. Rabbit polyclonal anti-CD36, -ERK1/2, -SRB1, -Nrf2, NFkB(p65) and Goat polyclonal anti-ABCG1,-LOX 1, -p38, -JNK and -PPARγ antibodies; horse-radish peroxidase conjugated rabbit-anti-goat and mouse-anti-rabbit antibodies, Benzamide and PARP inhibitor were procured from Santacruz biotechnology. Rabbit polyclonal LC3 I and LC3 II and cleaved PARP were obtained from cell signaling technology,USA. CD 36 inhibitor, Sulfo-N-succinimidyl oleate (SSO),was obtained from R&D Systems [Minneapolis, MN, USA]. Anti MMP 9 antibody (rabbit polyclonal to MMP 9) was obtained from abcam[USA], human MMP 9 standard from Millipore[USA] and Apo A1 antibody (rabbit polyclonal to Apo A1) from Santacruz..

3.2. Methods

3.2.1. Blood sample collection

Fasting blood samples [~10 ml] were collected from apparently healthy volunteers including blood donors, students and employees of this institute[total sample size~100] and clinically diagnosed CAD patients[total sample size~40] ,who

were admitted in the Cardiology ward of this hospital from both sexes in the age group of 25 to 55yrs. Brief clinical history of concerned risk factors was taken. Patients with diabetes mellitus were excluded as it is known to affect HDL functionality. All patients were receiving lipid lowering drugs and standard drugs like nitrates, β blockers and/or calcium channel blockers for the treatment of angina and hypertension. Prior informed consent was obtained for examining blood samples as per institute's research ethical guidelines (SCT/IEC-536/February2014). 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.

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

Cholesterol ester + H_2O Cholesterol esterase $\longrightarrow$ Cholesterol + fatty acid

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

2 H_2O_2 + 4- amino antipyrine + Phenol Peroxidase $\longrightarrow$ quinonamine+ 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, Singapore] and calculated the values against standard.

3.2.2.2. Estimation of triglycerides in serum

Serum triglycerides were quantitated by the recommended enzymatic method (GPO-POD) using reagent kits.

Triglycerides in serum was determined after enzymatic splitting with lipoprotein lipase(LPL). 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 ⟶ glycerol + fatty acids

Glycerol + ATP + Glycerol 3 Kinase ⟶ glycerol- 3-phosphate + ADP

Glycerol- 3 –phosphate + O_2 + Glycerol 3 phosphate oxidase ⟶ Dihydroxy acetone phosphate + H_2O_2

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

Procedure : 1 ml reagent was mixed with 10 µl of serum or standard and 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.

3.2.2.3. Quantitation of LDL-C in serum

Serum LDL-C was calculated from triglycerides, total cholesterol and HDL-C using Friedewald formula (Friedewald et al., 1972) as LDL-C= total cholesterol-(TG/5+HDL-C) mg%

3.2.3. Isolation of HDL (d= 1.063- 1.21 g/ml) by ultracentrifugation

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

2. Solution 2- 1.54% NaCl , density 1.36 g/ml

Procedure: Fresh serum was collected from healthy subjects and subjected to sequential ultracentrifugation using Beckman Optima TLX 120 ultracentrifuge with a fixed angle rotor type 120.2 according to Beckman application note -Bioresearch DS-693-Lipoprotein separation. 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 4,36,000xg 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%)

3.2.3.1. Isolation of HDL by PEG and estimation of HDL-Cholesterol

Reagents: 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[modified method of (Izzo et al., 1981)]. 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.

3.2.3.2. Electro elution of HDL

Electroelution of lipoproteins from acrylamide gel was done according to the method of Ogden and Adams(Ogden and Adams, 1987). Proceeding PAGE, the gels were taken out of the glass tubes and washed with distilled water. One of the gels was then stained for protein with Coommassie brilliant blue R-250 and destained. Using this gel as a marker, individual bands were sliced from the non stained gels and equilibrated in 1 to 2 ml Tris-acetate buffer (0.01 M, pH 8) for 15 min and homogenized using a Potter-Elvehjem homogenizer. Protein in suspensions were electroeluted into the buffer by immersing the bags containing the homogenized gels between opposite electrodes in a rectangular reservoir with 2 L of cold Tris-acetate buffer and applied a constant voltage of 100 V across the electrodes for 3 h. The bags were then dialyzed against PBS, containing 0.1% EDTA and 0.01% sodium azide and the gel pieces were pelleted by brief centrifugation at 3000 x g. The protein concentration of the eluted lipoprotein was estimated by lowry's method(Lowry et al., 1951)

3.2.4. Isolation of LDL(d= 1.019-1.063 g/ml) by ultracentrifugation

Reagents: 1. Solution 1- 0.9% NaCl, 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) was removed. 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 collected 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.

3.2.4.1. 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 $CuSO_4$ 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 bovine serum albumin(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.

3.2.4.2. Polyacrylamide disc gel electrophoresis(PAGE) of isolated lipoprotein

(Oya F, 1974)

Reagents

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 (DW), pH 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 were dissolved in 15 ml distilled water 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 were dissolved in 15 ml distilled water 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.

Procedure: 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 concentrating 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 5mA/tube. The gels were visualized for resolution of lipoproteins and scanned with an Image scanner (Amersham Biosciences, USA).

3.2.4.3. Assay of antioxidant capacity of HDL (Functional assay)

The inhibition of LDL oxidation is a major antiatherogenic property of HDL.The functionality of HDL was assessed in terms of the antioxidant capacity as apparent from its ability to prevent the formation of the fluorescence signal generated by oxidized LDL using cell-free assay, where DCFH-DA[Dichlorodihydro-fluorescein diacetate] was used as a fluorescent probe(Navab et al., 2001).

LDL oxidation activates DCFH fluorescence. Pooled LDL fraction was prepared from normal serum by ultracentrifugation as previously described. Briefly an aliquot of LDL at a concentration of 250 µg LDL-C/ml was subjected to air-

oxidation for 2 h in the presence and absence of HDL at a concentration of 350 µg HDL-C/ml and then treated with 10µl of DCFH-DA (2 mg/ml) for 1 h and the resultant fluorescence was measured (EX:485 nm/EM:530 nm) using Fluorescence Elisa Plate Reader (Biotek FLX 800). The assay distinguishes the antioxidative potential of HDL taken from different persons. Generally HDL isolated from healthy volunteers shows remarkable antioxidant capacity to inhibit LDL oxidation and is termed 'functional' HDL[nHDL].When the HDL fails to inhibit LDL oxidation or do not perform its biological tasks then it is termed 'dysfunctional' HDL[proinflammatory HDL, piHDL] (Smith, 2010a). Antioxidant acapacity of HDL was expressed as % of inhibition of LDL oxidation.

3.2.4.4. Assay of serum lipidperoxides (TBARS assay)

(Ohkawa et al., 1979)

Reagents: (1) 8.1 % SDS (2) 20 % acetic acid (3) 0.8 % Thiobarbituric acid(TBA) (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 95^0C 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 as 1.56×10^5 $m^{-1}cm^{-1}$.

3.2.4.5. 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 carbonyls were quantitated by the method described by Levine et al.(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 mixture (1:1) to remove lipids and excess DNPH. The precipitate was dissolved in 6 M guanidine hydrochloride and the absorbance was recorded at 370nm in UV-VIS spectrophotometer. The data were expressed as µM of carbonyl groups/L serum by using a molor absorption coefficient of 21,000 M^{-1} cm^{-1} for the DNPH derivatives.

3.2.4.6. Assay of serum paraoxonase activity [PON-1]

(Lorentz 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}.

3.2.4.7. 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 sulphuric acid. The absorbance was recorded after 10 minutes at 450nm in an ELISA microplate reader.The amount of colour developed is directly proportional to the concentration of CRP.

3.2.5. Monocyte cell isolation and culture

Human peripheral blood mononuclear(HPBM) cells were isolated from blood of healthy volunteers using Histopaque 1077 based density gradient centrifugation according to manufacture’s instruction. Briefly, 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^oC. 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 morphology of the cells was monitored under microscope and immunocytochemistry was carried out for cell specific marker, CD14. 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.

3.2.5.1. Monocyte differentiation to macrophages

Monocytes were isolated from blood as described above. 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^oC. For differentiation to macrophages, the non adherent monocytes were removed by washing the wells twice with RPMI, and the monocytes adhered to dishes were grown in the culture medium supplemented with 10% autologous human serum, penicillin (100 U/I), streptomycin (100 mg/l), and gentamicin (100 mg/l) for 8 days. The medium was replaced every two days. The morphology of the cells was monitored under microscope and immunocytochemistry was carried out for cell specific marker, CD68.

3.2.5.2. 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 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%.

3.2.5.3. MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay

Effect of HDL treatment on monocytes viability was determined by the MTT assay(Wilson, 2000). It is based on the ability of viable cells to reduce the MTT to insoluble formazan crystals by mitochondrial dehydrogenase. The cells were treated with MTT (final concentration 0.5 mg/ml) in RPMI without Foetal Bovine Serum(FBS) and incubated for 4 hours. After incubation, DMSO was added to dissolve insoluble crystals and the absorbance was read at 570 nm using microplate reader (Biotek ELX 800). Results were expressed as percentage of cytotoxicity:

$$\text{Percentage of Toxicity} = \frac{\text{Absorbance of Control - Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

3.2.5.4. Cell treatment

Cells were serum starved overnight and maintained in culture as described above and then treated with a medium containing PBS alone or with HDL (functional HDL or dysfunctional HDL) at varying concentrations 10 to 200 µg protein/ml and cultured for 24 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.

3.2.5.5. Measurement of intracellular reactive oxygen species (ROS)

Intracellular reactive oxygen species were measured by DCFH method (Liu etal ,2009). 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 (2mg/ml DMSO)

(2) Working DCFH – 10µl aliquot(20µg/10µl)

Procedure: Monocytes–macrophages after treatment with HDL were incubated with DCFH-DA 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.

3.2.5.6. Assay of tumour necrosis factor- alpha(TNF-α)

Concentrations of TNF-α in cell culture supernatants were determined using enzyme linked immunosorbent assay (ELISA) kits according to manufactures 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 and again incubated for 30 min at room temperature. Then 3,3',5,5'-Tetramethylbenzidine[TMB] 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).

3.2.5.7. Assay of interlukin 10 (IL-10)

Concentrations of IL-10 in cell culture supernatants were determined using ELISA kits, 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 IL 10 and incubated for half an hour at room temperature. After washing the wells,100 µl of biotinylated antibody reagent was added and incubated for one hour at room temperature. After washing the wells, 100 µl of streptavidin-HRP reagent was added and again incubated for 30 min at room temperature. Then TMB 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).

3.2.5.8. Activity of Gelatinases or matrix metalloproteinases (MMP-2& MMP-9)

Activity of MMPs secreted by the cells to the culture medium was determined by gelatin zymography(Toth et al., 2012). For this, 7.5 % SDS-PAGE polymerized together with gelatin (1 mg/ml) was used. Briefly, Cell culture supernatants, normalized to an equal amount of protein (60 µg), 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 24hr. 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).

3.2.5.9. Oil Red O staining for neutral lipid accumulation

After treatment, 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. Cells were then counter stained with haematoxylin [100mg/l] for 5 minutes.Stained cells were examined under a microscope [IX51 inverted basic microscope, Olympus, Japan] at 20 X magnifications. Cells with more than 6 or large Oil Red O-positive droplets were counted.

3.2.5.10. Quantitation of cellular cholesterol

After treatment, lipids were extracted from cells using 1ml hexane:isopropanol (3:2,v:v)(Robinet et al., 2010). Briefly, 1ml of the hexane:isopropanol mixture was added to cells in a 30mm culture plate. After incubation for 10min the cell extracts were transferred to microfuge tubes and dried. Cholesterol ester content of the dried extract was determined by CHOD-PAP method using enzymatic assay kits (Helix India) in Autochem NexGen analyser. Then the protein from the same well was dissolved by addition of 1.4 ml of 0.2N NaOH. The plate was incubated at 37°C for 3h. The protein lysates were transferred to microfuge tubes and protein concentration was quantified using Lowry method(Lowry et al,1951).

3.2.5.11. Propidium iodide staining for measuring cell death

Monocytes-macrophages after treatment were first stained with propidium iodide (PI stains dead cells only) and subsequently stained with cell-permeable Hoechst 33342 (stains all cells)(Serra-Perez et al., 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.

3.2.5.12. Estimation of glutathione peroxidase

The cellular activity of Glutathione peroxidase [GPx] was determined according to the method of Moron etal(Moron et al., 1979). GPx enzymes catalyze the reduction of hydrogen peroxide and a wide variety of organic peroxides (R-OOH) to the corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing reagent, which is then coupled to the oxidation of NADPH to NADP+. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is an indicative of GPx activity. Briefly, 50μl of cell lysates was added to 3ml of reaction mixture containing 1mg of NADPH,1mM sodium azide and 200mM reduced glutathione. The tube containing 3ml reaction mixture and 50μl of phosphate buffer was taken as blank. After mixing,

50µl of 0.042% of H_2O_2 was added to the reaction mixture and the absorbance was measured at 340 nm in a UV-visible spectrophotometer(UV-1601 PC, shimadzu). Enzyme activity was calculated as µ moles of glutathione utilized/ min/ mg protein.

3.2.5.13. Estimation of glutathione reductase

The cellular activity of Glutathione reductase was determined following the procedures of Mohandas et.al (Mohandas et al., 1984) by measuring the decrease in absorbance at 340 nm. The reaction mixture with a total volume of 2ml consisted of 1.6 ml of sodium phosphate buffer [0.1M, pH 7.4], 0.1 ml of EDTA [0.5mM], 0.05ml of oxidized glutathione[1mM], 0.1 ml cell lysate and 0.1 ml of NADPH [0.1mM]. The enzyme activity was quantified by measuring the disappearance of NADPH at 340nm for 3min at 30 sec intervals. The activity was calculated using a molar extinction coefficient of 6.22×10^3 $M^{-1}CM^{-1}$ and was expressed as µmol NADPH oxidized /min/mg protein.

3.2.5.14. Estimation of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity in macrophages , was estimated by the method of Bolann etal(Bolann and Ulvik, 1991). This assay was based on the ability of superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium by superoxide. Illumination of riboflavin in the presence of O_2 and electron donor like methionine generates superoxide anions and this has been used as the basis of assay of SOD. The reduction of NBT by superoxide radicals to blue coloured formazan was measured at 560 nm. Briefly, 100µl of cell lysate was added to the reaction mixture containing 50mM potassium phosphate buffer (pH 7.8), 45mM Methionine,

5.3mM riboflavin, 84µM nitroblue tetrazolium (NBT) and 20µM potassium cyanide. The tubes were incubated in 25^0C for 10 minutes and the absorbance of blue formazan was measured using a spectrophotometer at 600 nm. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions. Specific activity is expressed in terms of units per mg protein

3.2.5.15. Estimation of catalase activity

The catalase activity of the cell lysate was determined by adopting the method of Sinha etal(Sinha, 1972) . The assay is based on the decomposition of H_2O_2 by catalase, and determination of the remaining H_2O_2 by spectrophotometry at 530 nm. In brief, cell lysate(0.2ml) was added to a reaction mixture containing 1.0ml phosphate buffer (0.067M, pH 7) , 0.4 ml distilled water and 0.5 ml 0.2M H_2O_2 and allowed to stand for 60 minutes.The reaction was stopped by the addition of 2 ml of dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 minutes, cooled and the colour developed was read at 530 nm. The activity of catalase was expressed as µmol of H_2O_2 utilised / second).

3.2.5.16. Estimation of reduced glutathione

The cellular activity of reduced glutathione was estimated spectrophotometrically at 412nm which involves oxidation of GSH by thiol reagent, DTNB[5-5'-dithiobis 2-nitrobenzoic acid], according to the method of Beutler and Kelley(Beutler and Kelly, 1963). Reduced glutathione forms a yellow coloured complex [5′-thio-2-nitrobenzoic acid] with thiol reagent, DTNB[5-5'-dithiobis[2-

nitrobenzoic acid] with an absorbance at 412 nm. Briefly an aliquot [0.2 ml] of cell lysate was mixed with 1.8 ml of EDTA[1mM] and 3.0 ml of precipitating reagent :[1.67 g of metaphosphoric acid, 0.2 gm of EDTA disodium salt, 30 gm sodium chloride in 1 litre of distilled water] and kept for 5 minutes . After centrifugation, 2 ml of the filtrate, was added to a reaction mixture containing 4 ml of disodium hydrogen phosphate [0.3 M] and 1 ml of DTNB(40 mg of DTNB in 100 ml of 1% sodium citrate) and the colour developed was read at 412 nm in a spectrophotometer. The GSH content of the sample was expressed as µmol/mg protein.

3.2.5.17. Comet (Single-cell gel electrophoresis) assay

The comet assay (single-cell gel electrophoresis) is a standard technique for detecting single/double-strand DNA breaks, alkali labile sites, DNA cross-links, base/ base-pair damages and apoptotic nuclei in eukaryotic cell(Singh and Stephens, 1997). The negatively charged DNA containing breaks, and broken ends were able to migrate toward the anode during a brief electrophoresis. It involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in alkaline pH conditions to form nucleotides containing supercoiled loops of DNA linked to the nuclear matrix, and electrophoresis of the suspended lysed cells. If the DNA was undamaged, the lack of free ends and large size of the fragments prevented migration. The comet head contains the high-molecular-weight DNA and the comet tail contains the leading ends of migrating fragments. Determination of the relative amount of DNA that migrated provided a simple way to measure the number of DNA breaks in an individual cell. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of

the comet tail relative to the head reflects the number of DNA breaks. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage. In brief, macrophage cell lysate(1×10^6) were mixed with 10µl of low melting point agarose and pipetted the cell suspension onto agarose-covered surface of a pre-coated slide. Once the cell suspension was solidified, normal melting agarose(NMA) was used as a final layer . After agarose has gelled [at 4^0C for 10 min], the slide was submerged in a covered dish containing cold lysis solution [2.5 M NaCL,100 mM Na_2EDTA, 10 mM Tris ,pH 10 and 1%SDS (containing 10% DMSO and 1%Triton X 100)], for 1 hour and then placed in electrophoresis buffer (300 mM NaOH and 1mM disodium EDTA, pH 13) for 20 minutes to allow unwinding of DNA. Electrophoresis was conducted at 300 mA for 20 minutes. After washing in neutralizing buffer (0.4 mM Tris, pH 7.5) the slides were stained with 50µl ethidium bromide(20µg/ml) and visualized using fluorescent microscope (Olympus CKX 41) and the captured comets were measured quantitatively using Tritek comet scoring software.

3.2.5.18. Mitochondrial membrane potential by flow cytometry

After treatment with HDL, macrophages were checked for Mitochondrial membrane potential by Muse MitoPotential Assay kit using MUSE analyzer. The assay allows for the simultaneous measurement of two important cell health parameters—change in mitochondrial potential (considered an early hallmark of apoptosis and cellular stress), and cellular plasma membrane permeabilization or cell death. Cellular energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane, and this accumulation

of energy in healthy cells creates a mitochondrial trans-membrane potential, ($\Delta\Psi m$) that enables the cell to drive the synthesis of ATP. Loss of the mitochondrial inner transmembrane potential is often,but not always, observed to be associated with the early stages of apoptosis. Collapse of this potential is believed to coincide with the opening of the mitochondrial permeability transition pores, leading to the release of cytochrome C into the cytosol, which then triggers the downstream events in the apoptotic cascade.Depolarization of the inner mitochondrial membrane potential is thus a reliable indicator of mitochondrial dysfunction and cellular health. The sssay utilizes the MitoPotential Dye, a cationic, lipophilic dye to detect changes in the mitochondrial membrane potential and 7-AAD as an indicator of cell death. High membrane potential drives accumulation of MitoPotential dye within inner membrane of intact mitochondria resulting in high fluorescence. Cells with depolarized mitochondria demonstrate a decrease in fluorescence and a downward shift. A dead cell marker (7-AAD) is also used as an indicator of cell membrane tructural integrity and cell death. It is excluded from live, healthy cells, as well as early apoptotic cells.

Procedure : Muse MitoPotential Dye 1:1000 in 1X assay buffer was added with 95ul of mitopotential working solution and was incubated at 37°C for 20 minutes. After incubation, 5 µL of Muse MitoPotential 7-AAD was added to each well containing 100µl cell lysate and mixed and further incubated for 5min at RT. The samples were loaded on to a Muse cell analyser for mitopotential analysis. Results from each run are stored in a data file, as well as its corresponding spreadsheet.

3.2.5.19. Apoptosis by flow cytometry

Cells after treatment were checked for apoptosis by Muse™ Annexin V & Dead Cell kit using MUSE analyzer, which allows quantitative analysis for live, early or late apoptosis or cell death. The Muse™ Annexin V & Dead Cell Assay utilizes Annexin V (a calcium dependent phospholipid binding protein) to detect phosphatidyl serine[PS] on the external membrane of apoptotic cells. PS is a membrane component normally localized to the internal face of the cell membrane. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V can readily bind them. A dead cell marker is also used as an indicator of cell membrane structural integrity. It is excluded from live healthy cells, early apoptotic cells as well as late apoptotic cells.

Procedure : 100µL of cells[1×10^5 to 1×10^7 cells/ml] containing fresh serum- or albumin-containing medium was transferred to separate tubes and treated with 100 µL of the Muse™ Annexin V & Dead Cell Reagent. The tubes were mixed thoroughly and incubated for 20 minutes at room temperature in the dark for staining. Then the cells were analyzed in a MUSE analyzer. Results from each run are stored in a data file, as well as its corresponding spreadsheet.

3.2.5.20. qRT-PCR analysis for CD36 expression

a) RNA isolation from monocytes-macropahges :

CD36 mRNA expression level in macrophages were assessed with quantitative reverse transcription – polymerase chain reaction, qRT-PCR. Total RNA was extracted from macrophages using TRIzol reagent (Invitrogen,USA) following the manufacturer's instruction**.** Monocytes – macrophages ($1x10^6$ cells/ml)

were incubated with HDL(50µg/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.

b) Assessment of purity of isolated RNA : The yield and purity of the isolated RNA was determined spectrophotometrically at UV absorbance A260 and A260/280, respectively. The intactness of RNA was ascertained by 1.5% agarose gel.

c) cDNA preparation from RNA for RT-PCR analysis: cDNA was generated by reverse transcription of 1 µg of total RNA using 100nM of dNTPs, 50pM of primer oligo dT15, 200U of M-MLVRT, 16U of protector RNase inhibitor in RT buffer and 2.5µl of 0.1MDTT with random hexamer primers following the protocol of the manufacturer. 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:

CD36 Forward primer‘ 5‘CTGGCAACAAACCACACACTGGA3‘

CD36 Reverse primer 5‘TGACAGCCCCAGCGATGAGC 3‘

GAPDH Forward primer 5‘ GTCGCCAGCCGAGCCACATC3‘

GAPDH Reverse primer 5‘ CGCCACAGTTTCCCGGAGGG 3‘

d) qRT-PCR analysis: PCR amplification protocol involved 30 cycles at 94°C, 30 s; 55°C, 30 s; 72°C, 30 s; and, finally, at 72°C, 5 min using 18S RNA as internal standard. GAPDH was used as an internal control for normalization of samples. The

amplified transcripts were analyzed by 2% agarose gel electrophoresis. Another set of experiment carried out in a similar way was amplified using SYBR Green in a detection system. A TaqMan quantitative RT-PCR analysis was carried out using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, CA) using SYBR Green (SYBR Green Master Mix, Invitrogen).

3.2.5.21. Western blot analysis

Western blot analysis was carried out by the standard protocol described by Maniatis etal(Maniatis, 1982). Briefly, cells after treatment were lysed in cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton] followed by incubation at 4ºC for 30 minutes. The lysates were centrifuged at 13,000 rpm for 30 minutes to remove cell debris, and the supernatant was aliquoted and stored at –80ºC until use. Protein was quantified by the lowry's method(Lowry et al., 1951). The lysates were denatured by incubation with 0.375 M SDS-gel loading buffer [9% SDS containing bromophenol blue (0.03%), β-mercaptoethanol (9%), glycerol (50% v/v) in 18.75 ml 1M Tris HCl- pH 6.8] at 100ºC for 5 minutes. 60 µg of protein was electrophoretically fractionated on 10% SDS-PAGE minigels and electroblotted onto nitrocellulose membrane for 90 minutes at 100V. The membrane with the transferred proteins (ascertained by Ponceau S staining) was blocked for 1 hour with 5% skim milk in Tris-Buffered Saline and Tween 20(TBST) and incubated overnight at 4ºC with primary antibodies prepared at a dilution of 1:1000 in TBST containing 5% BSA . Unbound primary antibody was removed by washing (5 min x 5 times) with TBST (1X TBS containing 0.1% Tween-20). Immunoblots were exposed for 1 hour to HRP-conjugated secondary antibody at 1:5000 dilution in

5% BSA-containing TBST . Protein bands were detected using the chemiluminescent ECL plus Western blotting detection system (Thermoscientific,USA) according to manufactures instructions, with average exposure time of 30 seconds to 15 minutes and image was scanned using image scanner (Amersham Biosciences). GAPDH and beta actin was used as loading control. Protein expression was quantified by densitometric scanning (Image J,NIH).

3.3. Statistics

Statistical analysis was carried out by GraphPad prism statistical software. Statistical difference between two sets of data was determined using unpaired Student's 't' test. One way ANOVA was employed to analyze the variation among groups. The correlation coefficient (r) was also used to assess the strength of any association between different variables. 'p' value of less than 0.05 was considered statistically significant.

RESULTS

4.1. Identification of the prevalence of functionally altered HDL in apparently healthy subjects and in pateints with clinically diagnosed coronary artery disease

Fasting blood samples were collected from clinically diagnosed CAD patients who were admitted in the Cardiology ward of this hospital and apparently healthy volunteers for isolation of HDL fractions for studying the functional properties. The sample group involves both sexes in the age group of 25 to 55yrs. The basic characteristics of the subjects studied are provided in Table I.

Table 1: Basic characteristics of study subjects

	Healthy subjects	CAD patients
Total sample size	100	40
Sex (Males/Females)	37/63	18/22
Body mass index(Wt in Kg/ Ht in m^2)	22±3	25±4*
Serum cholesterol (mg/dl)	225±41	146±20*
Triglycerides (mg/dl)	85±15	137±17*
HDL-Cholesterol (mg/dl)	50±9	32±8*
LDL-Cholesterol (mg/dl)	120±14	87±10*
Glucose (mg/dl)	92±15	93±12

Values are the mean±SD, * 'p'<0.0001.

All the CAD patients were on standard antianginal drugs and statins, that result in lower levels of total cholesterol and LDL-C, compared to controls.

4.1.1. Isolation of HDL and LDL

In order to study the functionality, HDL and LDL fractions[from controls only] were isolated from blood samples by sequential ultracentrifugation. The isolated lipoproteins were dialyzed against PBS at 4 ^{0}C and the purity was checked by polyacrylamide disc gel electrophoresis (3.75% PAGE).

4.1.2. Purity of isolated lipoprotein

The purity of isolated lipoproteins was checked with PAGE and the results are given in Fig. 6. HDL fractions isolated by ultracentrifugation resolved as a single band in PAGE and confirmed the absence of other lipoproteins, i.e. LDL and VLDL.

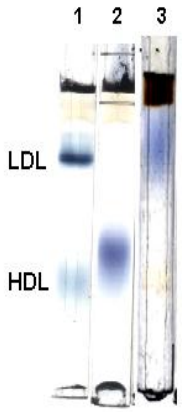


Figure 6: Disc gel electrophoretic pattern of serum lipoproteins on 3.75% polyacrylamide gels. *lane 1- serum, lane 2- ultracentrifugally isolated HDL fraction, lane 3- ultracentrifugally isolated LDL fraction.*

4.1.3. Antioxidative capacity of HDL in healthy subjects and in CAD patients

About 10 ml of blood was collected from patients with established CAD [n= 30]. The control group consisted of equal number of age and sex matched apparently healthy volunteers. HDLs were isolated from serum by sequential ultracentrifugation and subjected to functionality assay, where DCFH- DA was used as a fluorescent probe. The fluorescence intensity observed for LDL oxidation in the presence and absence of a test HDL [nHDL or piHDL] is presented in Fig. 7.

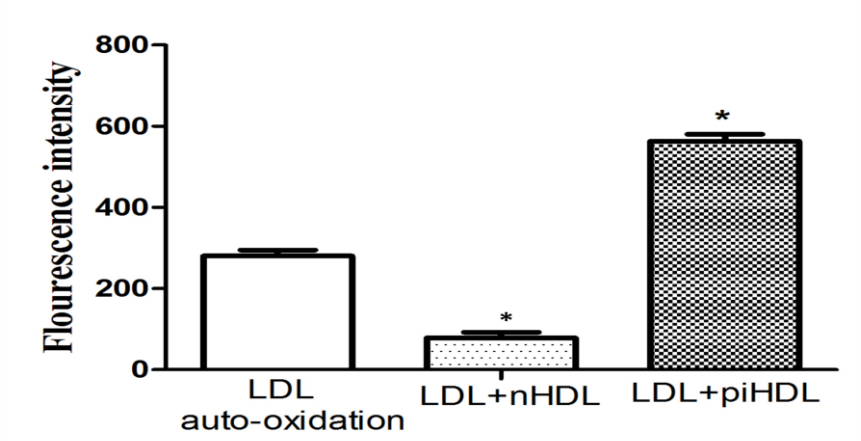


Figure 7: Antioxidant capacity of HDL from healthy subjects[nHDL] and piHDL against LDL-oxidation. *An aliquot of control LDL(250µg LDL-C/ml) was subjected to air-oxidation for 2 hour in the presence and absence of nHDL or piHDL(350µg HDL-C/ml) and treated with 10µl DCFH-DA(2mg/ml).The resultant fluorescence was recorded in Fluorescence Elisa Plate Reader.Values are the mean±SD, *'p'*<0.001.

HDL from controls exhibited remarkable antioxidant property as it significantly inhibited LDL oxidation [% inhibition of LDL oxidation=70% ± 14, 'p''< 0.001]. However, HDL from CAD patients did not possess antioxidant activity and did not inhibit oxidation of LDL. On the contrary, it increased LDL oxidation to a greater extent as evidenced by enhanced fluorescence compared to LDL auto-

oxidation, demonstrating dysfunctionality in HDL as well as its pro-inflammatory nature.

Further, the inflammatory index of HDL[iiHDL] was calculated in a subgroup of 30 pairs,[nHDL and piHDL] where the subjects within each pair have almost same level of HDL-C but they were functionally different. For this calculation, the fluorescence of oxidized LDL was used as an index [by normalizing LDL oxidation to 1.0] and examined the influence of HDL on this effect. The proinflammatory HDL (piHDL) was defined by an HDL inflammatory index greater than 1.0 and that with less than 1.0 was defined as anti-inflammatory HDL [Fig.8]. nHDL from healthy subjects showed an inflammatory index of 0.39±0.34, indicating anti-inflammatory nature. But, piHDL as a group was found to be pro-inflammatory as evidenced by an inflammatory index of 1.64±0.57[*'p'<0.01].

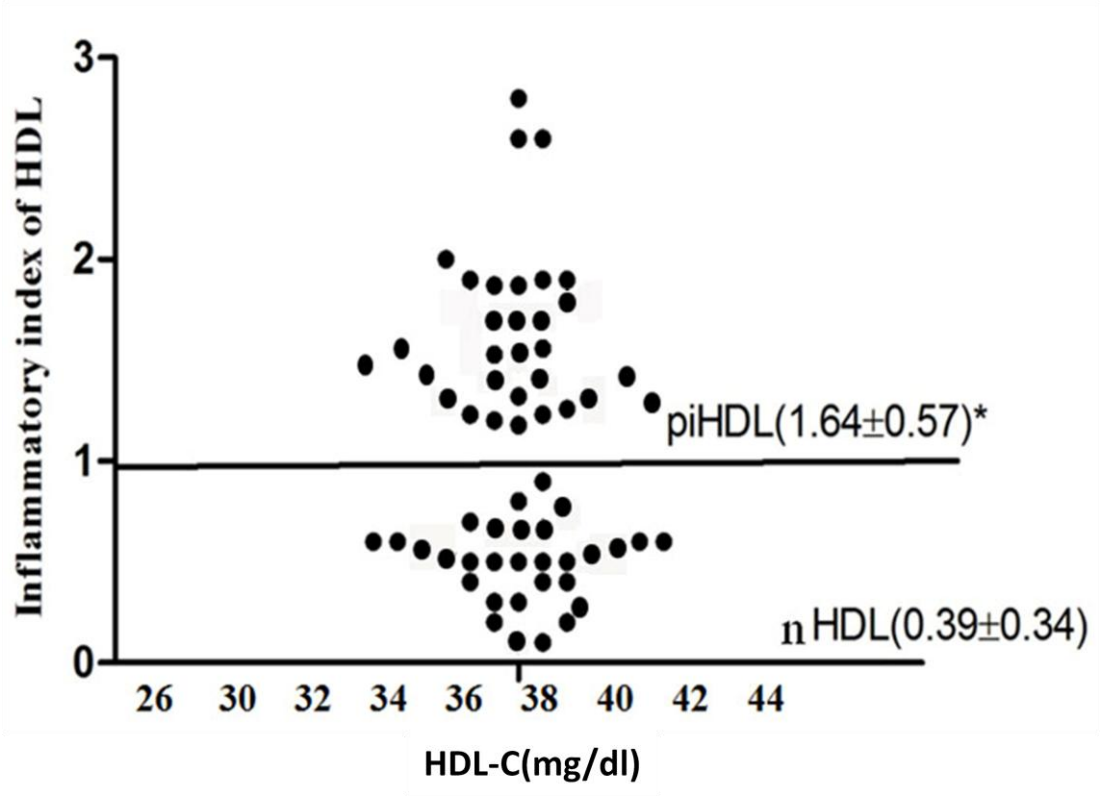


Figure 8: Inflammatory index of HDL calculated using the fluorescence of oxidized LDL as an index. *Each dot represents inflammatory index of HDL . The horizontal line at 1.0 indicates LDL oxidation index (>1.0 is pro-inflammatory HDL).*p<0.01*

When the functional status of circulating HDL was assessed among healthy volunteers using larger sample size[n=50], it was observed that HDL functionality varied widely irrespective of the level of HDL-C[Fig. 9]. For e.g. HDL isolated from five subjects having same levels of HDL-C,[32 mg%],when tested for functionality, they showed different anti-oxidant capacity to inhibit LDL oxidation – 30%, 47%, 55%, 64% and 79%. Similar finding was observed when tested for other levels of HDL-C as indicated in Fig.9. It was also interesting to observe zero percentage (0%) antioxidative capacity for HDL[i.e.HDL did not possess any antioxidant property], among healthy subjects [in three cases] even after having normal serum HDL-C

concentrations of 40, 46 and 49 mg/dl indicating the prevalence of dysfunctional HDL. These findings indicated that the functional capacity of HDL varied among healthy subjects irrespective of the levels of HDL-C and the levels of HDL-C did not show any relation to the antioxidative property of HDL (r=0.15).

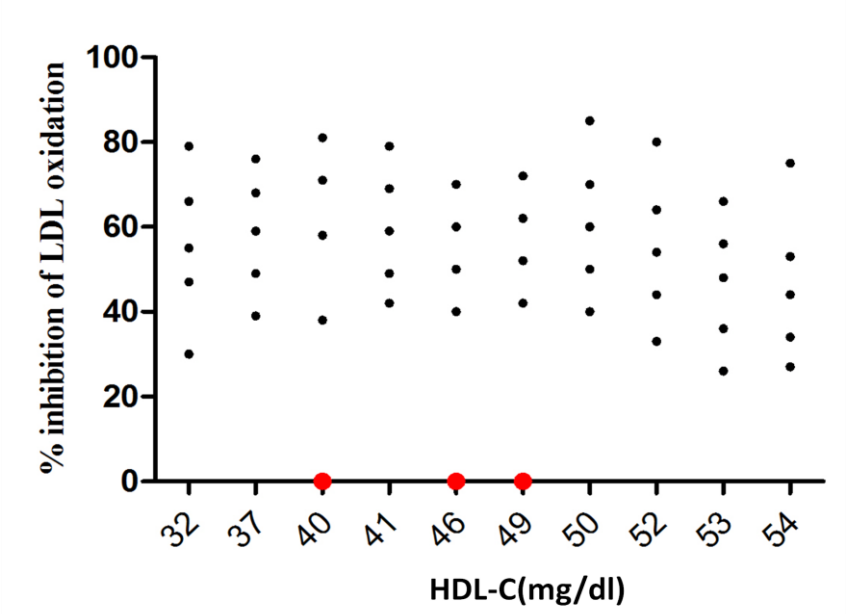


Figure 9: Antioxidative capacity of HDL isolated from healthy volunteers. *Functional assay of HDL was carried as described above. Antioxidative capacity of HDL is expressed as % inhibition of LDL oxidation.Each dot in a row represent antioxidative capacity of HDL from five different individuals matched for HDL-C.*

4.2. HDL Functionality and Serum Lipid Parameters

It is well established that an atherogenic lipid profile is associated with increased cardiovascular risk. To examine whether dysfunctionality in HDL showed any association with the lipid parameters, serum levels of total cholesterol, triglycerides (TG), LDL-C,HDL-C and phosohlipids were quantitated in three groups of subjects having functional HDL and dysfunctional HDL[Fig 10]. Healthy subjects[n=50] were grouped according to the functionality of HDL, as having nHDL [group A], and piHDL [group B]. A third group consisting of HDL from CAD

patients with piHDL.Total cholesterol and LDL-C were significantly higher in group B with piHDL compared to group A[nHDL], and group C[piHDL]. In group B and C with piHDL, a significant increase in the levels of TG and phospholipids was observed than that of group A with nHDL ['p'<0.05]. However, the level of HDL-C was found lower['p'< 0.05] only in CAD patients[in group C], with piHDL. Among healthy subjects, no difference was observed in the mean levels of HDL-C between subjects with nHDL[group A] and piHDL[group B].These results showed that subjects with piHDL have higher levels of serum TG and phospholipids compared to those having nHDL.

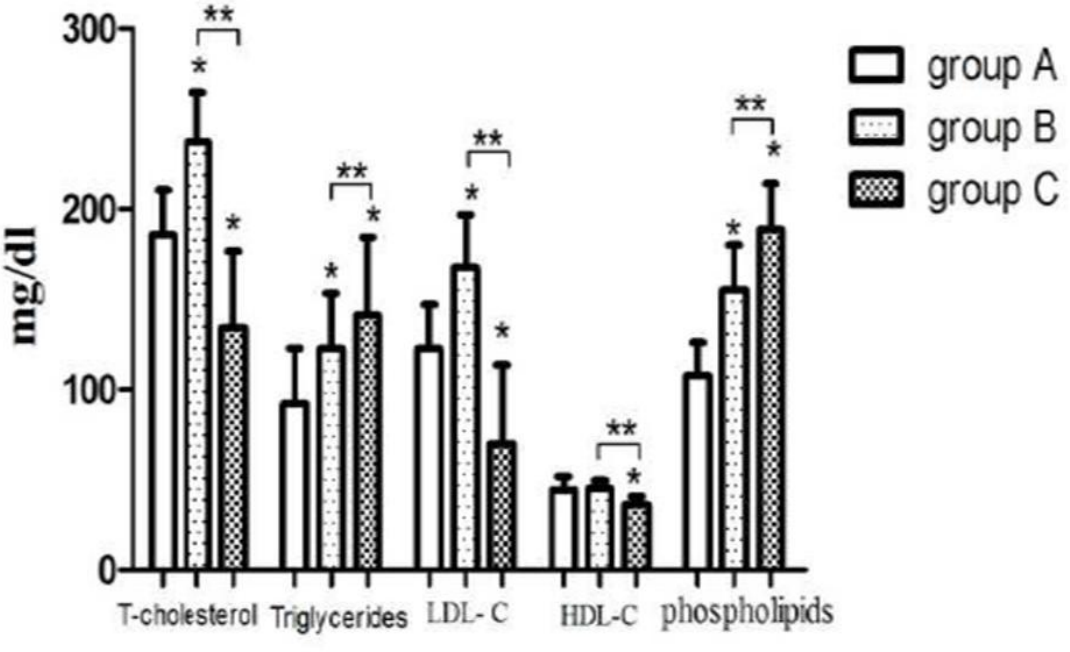


Figure 10: Lipid profile of subjects having functional and dysfunctional HDL. *Healthy subjects with nHDL(group A,n=47) and piHDL (group B,n=3),CAD patients with piHDL(group C,n=30). Values are the mean ±SD.*p<0.05 A vs B & C, **p<0.05 B vs C.*

4.2.1. HDL Functionality and systemic oxidative Stress markers

Oxidative stress represents an imbalance between prooxidants and anti-oxidants. HDL has an array of antiatherogenic mechanisms, which prevent the formation and promote removal of lipid peroxides from other proatherogenic lipoprotein and cell membranes. It is not clear whether dysfunctionality in HDL has any association with systemic oxidative stress. Hence the serum markers of oxidative stress- such as lipid peroxides and protein carbonyls, and the antioxidant- vitamin C, were quantitated in subjects having functional and dysfunctional HDL.It was observed that the levels of both lipid peroxides and protein carbonyls were significantly higher in subjects with piHDL [group B & C] than those with nHDL, [Fig.11]. Further, Vitamin C content was found decreased in subjects with piHDL and the difference was more significant in CAD group C[vit.C content in group:A =1.38±0.41;B=0.83±0.27; C= 0.57±0.22 mg/dl, p <0.01]. These results suggest the association of dysfunctionality in HDL with systemic oxidative stress.

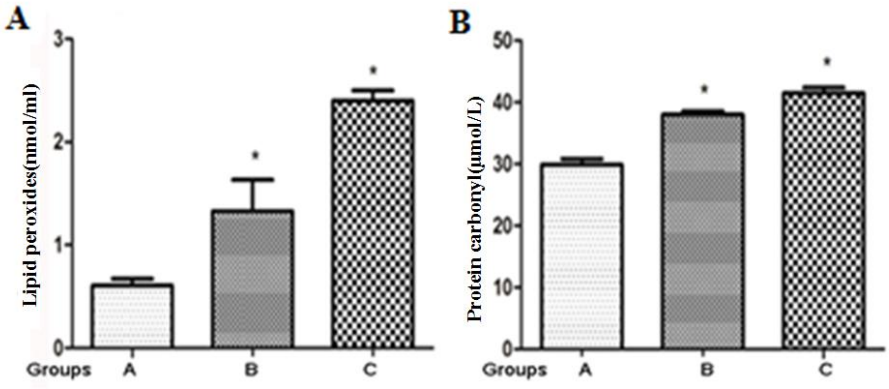


Figure 11: Systemic oxidative stress status of subjects having functional and dysfunctional HDL.*(11a) lipid peroxides expressed as malondialdehyde ,(11b) protein carbonyls. Values are expressed as mean ±SD.*p<0.05*

4.2.2. HDL functionality and systemic inflammation

Inflammation is a major contributor to atherogenesis. To study the systemic inflammatory status, serum hs-CRP and MMP 9 were quantitated in these three groups of subjects and the results are presented in Fig.12. When compared to subjects with nHDL, subjects with piHDL had higher levels of hs-CRP, MMP 2 and MMP-9 activity, indicating the association of dysfunctionality in HDL with systemic inflammation.

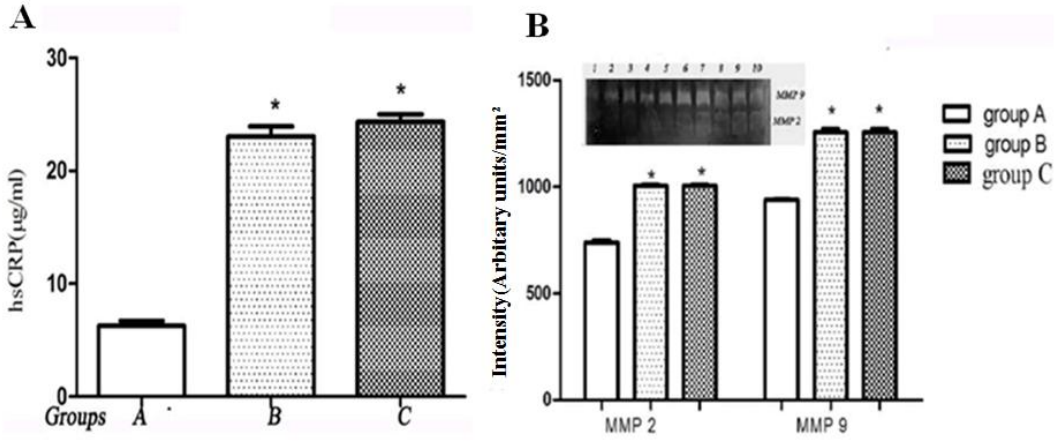


Figure 12: Systemic inflammatory status of subjects having functional and dysfunctional HDL.(*12a)serum hsCRP,(12b)gelatinase activity(MMP-2 & MMP -9). Lane:1to5- nHDL;6 to10- piHDL. Values are expressed as mean ±SD.*p<0.05*

4.2.3. Characterisation of isolated HDL

HDL undergoes functional alteration in subjects under inflammatory condition. To determine the associated compositional changes, HDL fraction was isolated from serum by ultracentrifugation and subjected to compositional characterization and the results are presented in Table II. The lipidomic analysis

showed the enrichment of TG, phospholipids, and lipid peroxides in piHDL[group B & C] in comparison to nHDL[group A]. Cholesterol content of HDL particle was found decreased only in group C, CAD patients. However, no difference was observed in the mean levels of cholesterol between nHDL and piHDL [group A & B] in healthy subjects. Further, piHDL particles showed decreased activity of paraoxonase-1,an antioxidant enzyme and the difference was found more significant in CAD patients (group C), compared to nHDL [group A]. The relative resistance of HDL particle to in vitro air-saline auto-oxidation[for 2 hour] was also assessed by measuring the formation of ROS as DCF fluorescence. A marked reduction in resistance to auto-oxidation was observed for piHDL particle, as evidenced by enhanced ROS formation than the nHDL particle.

Table II:Characterization of isolated HDL particle

PARAMETERS	**Group A** Subjects with nHDL[n=47]	**Group B** Subjects with piHDL[n=3]	**Group C** subjects with piHDL [n=30]
HDL-Cholesterol [mg/dl]	45.4 ±7.4	45.0±3.74 $^{\#}$	30.8±6.3*
HDL-Triglycerides[mg/dl]	37.8±6.1	67.8±4.1* $^{\#}$	86±7.8*
HDL-Phosholipids[mg/dl]	55.3±8.8	72.1±17.2* $^{\$}$	82.6±7.5*
HDL-Paraoxonase-1[ku/l]	137±23	87±4.6* $^{\$}$	57±15.2*
HDL-Lipid peroxides(nm/ml)	0.06±0.02	1.08±0.13*	1.08±0.09*
HDL-Susceptibility to auto-oxidation (ROS-fluorescence intensity)	77±10.3	112±8* $^{\$}$	136.2±12.16*

Values are the mean ± SD,∗ 'p' <0.001, group A vs. B & C; $^{\#}$ *'p'<0.001 &* $^{\$}$ *'p'<0.05 group B vs.C.*

4.3. Association of matrix metalloproteinase 9 (MMP 9) with dysfunctional HDL

Systemic inflammation can induce major changes in HDL composition and function. This study was carried out with the objective of examining whether dysfunctional HDL is associated with pro-inflammatory proteins other than the acute phase proteins as reported earlier. The above data indicated a significant association between dysfunctionality in HDL and systemic inflammation as evidenced by enhanced activity of MMP-9 and hsCRP. Gelatinase B (matrix metalloproteinase-9) is an important mediator of vascular remodelling, inflammation and atherosclerotic-plaque instability. Both HDL and apoB containing lipoproteins [VLDL,LDL and Lp(a)] were fractionated from serum using standard ultracentrifugation and then subjected to gelatin zymography. Fig.13 shows a panel of zymograms of lipoproteins isolated from controls and CAD patients exhibiting functional and dysfunctional HDL respectively. A consistent gelatinolytic band of MMP-9 was identified in all HDL fractions isolated from patients (Fig. 13A) but not in healthy controls as the latter has functional HDL. Further no detectable band of gelatinase activity was found in the apoB containing lipoproteins-VLDL, LDL and Lp(a), and lipoprotein-free serum fractions (Fig. 13B). To examine whether the dysfunctional HDL is associated with MMP 9, only 10 randomly selected samples each from healthy subjects and CAD patients were used for further experiments.

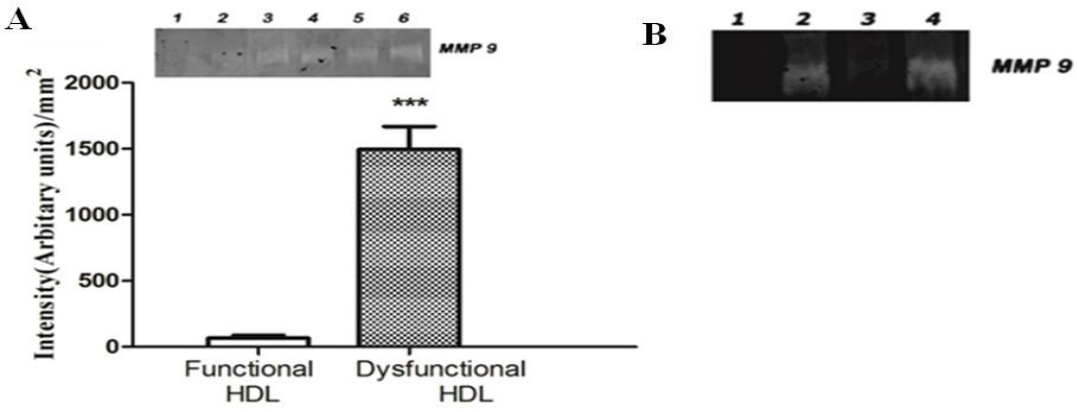


Figure 13: MMP 9 activity in isolated lipoproteins: *HDL- both functional and dysfunctional and apo B containing lipoproteins were isolated by UC and assessed for gelatinase activity using zymography . (13A) Inset shows the representative zymogram. Lane 1 & 2- functional HDL, lane 3, 4, 5 & 6- dysfunctional HDL. Densitrometric values are represented graphically. Values are the mean ± SD for 10 separate experiments. ***p < 0.0001 functional-vs. dysfunctional HDL (13B). Representative zymmogram of serum lipoprotein fractions from subjects having dysfunctional HDL. Lane 1- apoB containing lipoproteins [VLDL, LDL and Lp(a)]. Lane 2- serum without apo B containing lipoproteins and with HDL. Lane 3- lipoprotein-free serum [after separation of apoB containing lipoprotein and HDL], lane 4- dysfunctional HDL fraction.*

4.3.1. Association of MMP 9 with dysfunctional HDL

To confirm the association of MMP-9 with dysfunctional HDL, HDL fractions were isolated from serum using different methods-ultracentrifugation(UC), polyethylene glycol (PEG) precipitation, and electroelution (EE). Western blot, dot blot and zymography analyses of HDL [Fig. 14] confirmed the presence of a dominant 92 kDa protein corresponding to MMP 9, exclusively in the dysfunctional HDL fractions irrespective of the methods of isolation, indicating that dysfunctional HDL carries MMP 9. Further, the antioxidant property of these HDL fractions isolated by different methods were compared as assessed by DCFH-DA assay [Fig.

14D]. However, the oxidation rate was slightly higher ($p < 0.05$) when HDL was isolated by PEG precipitation method compared with other methods of isolation.

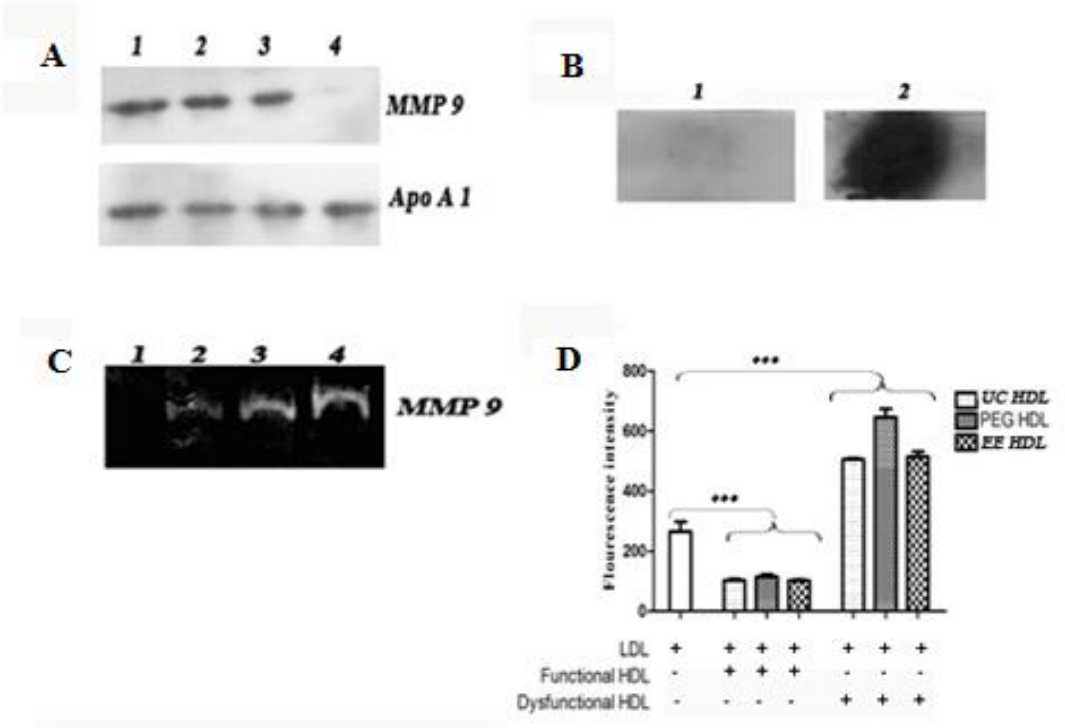


Figure 14: MMP 9 activity in HDL fractions isolated by three different methods and corresponding functionality of HDL. *(A) Western blot of HDL fractions probed with MMP 9 antibody(*rabbit polyclonal to MMP 9*). Apo A1 was used as internal control and detected with Apo A1 antibody(rabbit polyclonal to Apo A1) . Lane 1, 2, 3 dysfunctional HDL fractions isolated by UC(1), PEG(2) and EE(3)methods. Lane 4- functional HDL [UC]. (B) dot blot assay of EE HDL. Serum was subjected to PAGE in 3.75% gel. The band corresponding to HDL was excised, electroeluted and probed with MMP9 antibody. Lane 1&2- functional-and dysfunctional HDL respectively. (C) Gelatin zymogram of HDL isolated by different methods. Lane 1- functional HDL [UC], lane 2, 3, 4 dysfunctional HDL isolated by (2)UC, (3)PEG, (4)EE methods. (D) Antioxidant property of HDL. HDL isolated by three different methods. Values are the mean of 10 experiments ±SD, '***'$p < 0.0001$.*

4.3.2. HDL subfractions and MMP9 activity

Next aim was to evaluate whether one or both of the major HDL subclasses (HDL2, HDL3) in dysfunctional HDL exhibited MMP9 activity. HDL subfractions- HDL2 (density: 1.063-1.125 g/ml) and HDL3 (density: 1.125-1.21 g/ml) were isolated by ultracentrifugation, desalted and subjected to western blot and zymography analyses [Fig. 15]. Results from both analyses showed the strong association of MMP9 with HDL2 subfraction.

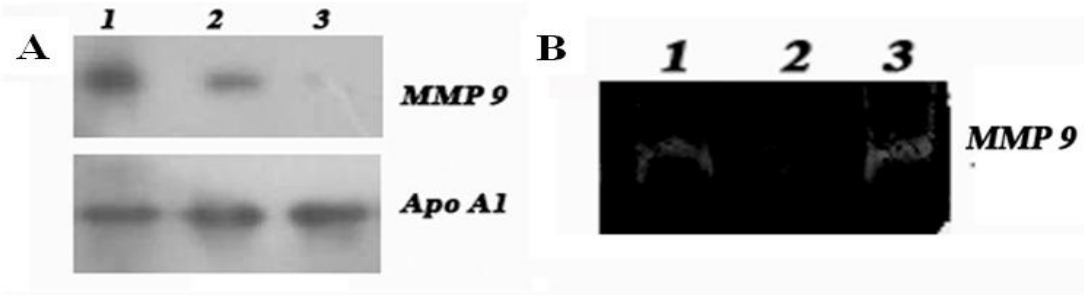


Figure 15: MMP 9 activity in HDL subfractions. *HDL and its major subfractions- HDL2(density1.063-1.125 g/ml) and HDL3 (density 1.125-1.21 g/ml) were isolated by ultracentrifugation and desalted. HDL fractions were subjected to western blot and gelatine zymography. (A) Western blot of HDL subfractions probed with MMP 9 antibody(*rabbit polyclonal to MMP 9*): lane 1- dysfunctional HDL, lane 2 &3- HDL2 and HDL3 subfractions of the same dysfunctional HDL. (B) Gelatin zymogram of HDL and its subfractions. Lane 1- dysfunctional HDL, lane 2 and 3- HDL3 and HDL2 subfractions of the same dysfunctional HDL showing gelatinolytc activity.*

4.3.3. Treatment of HDL with MMP9 and functional characterization of HDL

To examine the direct role of MMP-9 in inducing functional impairment in HDL, an invitro experiment was conducted where MMP9 was added to functional HDL. HDL [1 mg/ml] was incubated with MMP9 standard [1 μg/ml] in PBS at 37^0 C for 1 h. HDL was electroeluted and subjected to different assays. Gelatin

zymography of HDL showed a clear gelatinolytic band corresponding to MMP9 and was confirmed by western blot of HDL probed with anti MMP9 antibody, indicating the association MMP9 with HDL. In addition, MMP9 treated HDL also showed impaired antioxidant capacity, as the HDL enhanced fluorescence intensity [p< 0.001] in DCFH-DA assay. These results provide evidence that MMP9 can adversely affect HDL's ability to prevent LDL oxidation [Fig. 16] .

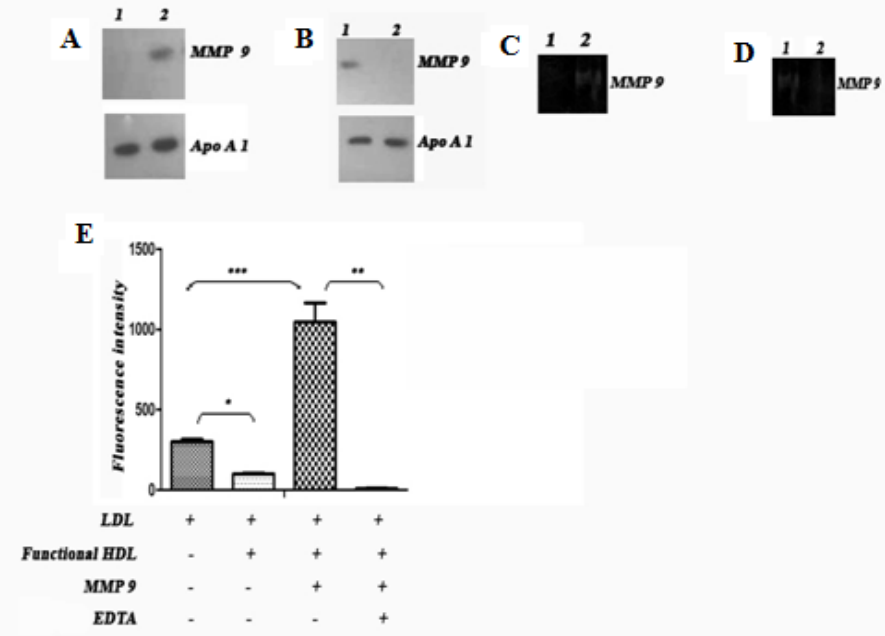


Figure 16: MMP 9 activity in HDL fraction pre-incubated with MMP9 and functionality of HDL. *Functional HDL was treated with MMP9 (A)Western blot of HDL incubated with MMP-9. lane 1- functional HDL without MMP-9 treatment, lane 2- functional HDL treated with MMP-9. (B) lane 1- functional HDL treated with MMP9 alone, lane 2- HDL after treatment with MMP9 exposed to EDTA (10 mM). (C) Gelatin zymogram of HDL treated with MMP-9. Lane 1-functional HDL without MMP9, lane 2- HDL treated with MMP-9. (D) Lane 1-HDL treated with MMP9 alone, lane 2- HDL after treatment with MMP9 exposed to EDTA (10 mM). (E) Functional property of HDL treated with MMP-9. LDL was subjected to air oxidation with and without native functional HDL or HDL treated with MMP9. values are the mean of 10 experiments ±SD, '***'p < 0.0001.*

4.3.4. Proteolytic activity of MMP 9 on apo A1/HDL

To examine whether MMP-9 caused any proteolysis, HDL treated with MMP-9 was analyzed by SDS-PAGE [10%]. The data presented in [Fig. 17] clearly revealed no degradation of apoA1due to the protease activity.

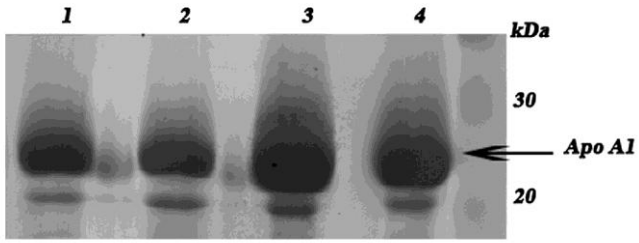


Figure 17: Proteolytic activity of MMP 9 on Apo A1. *Functional HDL fractions were incubated with MMP 9 standard as described above. Aliquots were electrophoresised in 10% SDS-PAGE. Lane 1, 3- functional HDL fraction without MMP 9, lane 2, 4- same HDL fraction treated with MMP 9.*

4.4. Dysfunctional HDL induces proinflammatory response

4.4.1. Monocyte isolation and 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 and the immunocytochemical analysis showed the predominanace of CD14 expression [Fig 18]. Trypan blue exclusion test was employed to assess the viability of cells and was found to be greater than 95%.

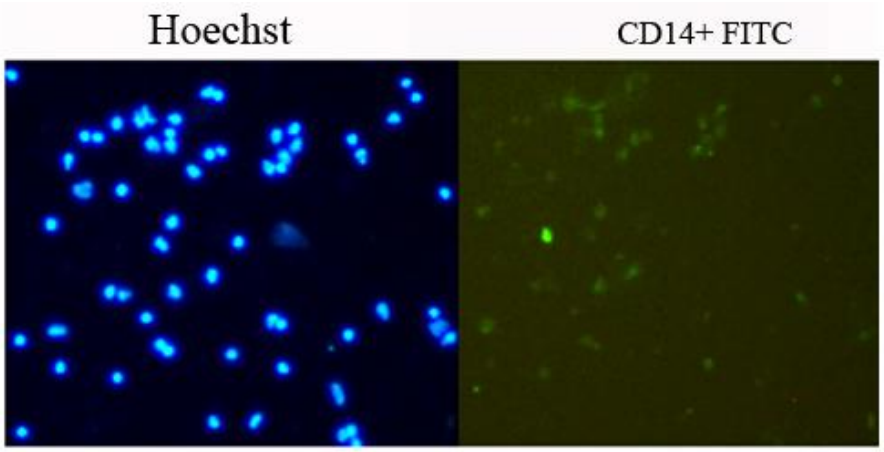


Figure 18: The CD14+ cells(monocytes). *PBMC cells ($1x10^6$) were stained with FITC labelled CD14 marker and fluorescent image was taken with Hoechst as counter stain (blue and green filter) under fluorescent microscope at 20 X magnification(IX 51 inverted basic microscope, Olympus, Japan). A- Hoechst staining of cells. B- corresponding FITC image showing CD14+ cells.*

4.4.2. Cell viability

Effect of nHDL and piHDL treatment on monocytes viability was determined by the MTT assay. Briefly, monocytes(1×10^6/ml) plated in 96 well plate were treated with nHDL and piHDL at various concentrations starting from 10µg/ml to 200µg/ml for 24h. Following incubation cytotoxocity was determined using MTT as described in methodology section. Results [Fig 19] indicates that nHDL was not toxic even upto a concentration of 200µg/ml and maintained cell viability[around 90%] after 24hr incubation. piHDL at a concentration of 50µg/ml and 70µg/ml was found to be less toxic [cell death less than 10%] compared to 200 µg/ml,at 24h. For further cell culture studies HDL at a concentration of 50µg/ml was used.

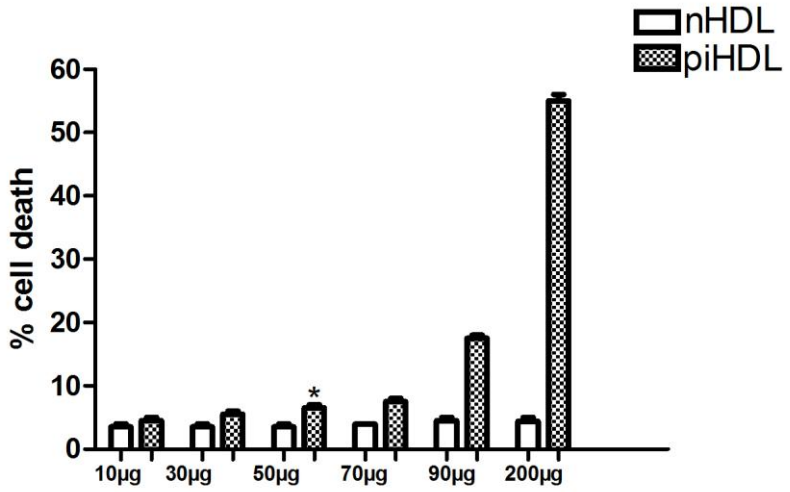


Figure 19: Cell viability by MTTassay. *Monocytes (1×10^6/ ml) were cultured under standard condition and were treated with dfferent concentration of nHDL or piHDL for 24h and subjected to MTT assay as described in methodology. The absorbance was taken at 540 nm in microplate ELISA reader(Biotek ELX 800).*

4.4.3. Pro-inflammatory response:

Monocytes/macrophages were incubated separately with dysfunctional HDL from CAD patients, MMP9-treated HDL or HDL after treatment with MMP9 exposed to EDTA (10 mM) for 24 h to see the effect of HDL on release of TNF-α, a marker of inflammation and IL 10, an anti-inflammatory marker, in comparison to functional HDL. It was observed that both dysfunctional HDL and MMP9-treated HDL induced greater generation of TNF-α than functional HDL from monocytes/macrophages. Furthermore, IL-10 formation in these cells was found to be decreased significantly, when exposed to dysfunctional forms of HDL ($p < 0.01$), suggesting the pro-inflammatory nature of dysfunctional HDL. On the contrary,

functional HDL stimulated more generation of IL-10, revealing its protective anti-inflammatory behaviour [Fig. 20].

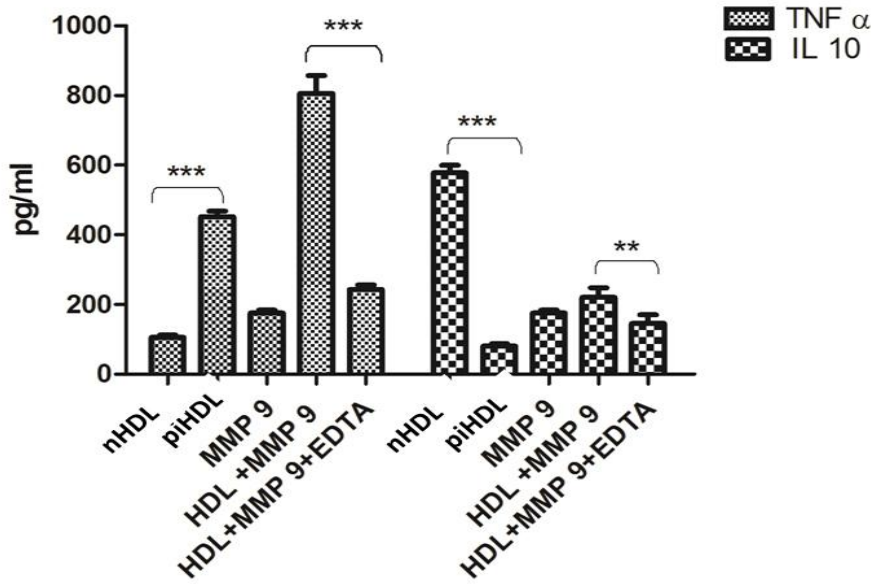


Figure 20: Monocyte/macrophage cytokine release induced by HDL. *Cells (1×10^6cells/ml) were cultured under standard condition and treated with functional HDL , dysfunctional HDL , MMP 9 standard,MMP9-treated HDL or MMP9 treated HDL after exposure to EDTA , for 24 h as described in methods. Levels of cytokines-TNF-α and IL-10, in the culture medium were quantitated using respective ELISA kits. Values are the mean of 10 independent experiments ±SD, '***'p <0.0001.*

To my knowledge this is the first report to demonstrate the previously unrecognised association of MMP 9 with dysfunctional HDL. This data is highly significant as it provides new insights in to the pro inflammatory property of HDL [results were published in *Atherosclerosis(2014), 236: 162-168*].

4.5. Biomolecular modeling showing binding of MMP9 with HDL

It has been recognized that HDL serves as a docking station for large number of proteins with variable binding affinities. Circulating HDL, although heterogeneous

in size and lipid composition are always found with apo A1 as the predomiant protein core. Here this study was aimed at investigating the molecular interaction between MMP-9 and HDL, based on insilico procedures using computer-aided molecular modelling and docking techniques. All docking studies were carried out at Department of Computational Biology and Bioinformatics, (University of Kerala), using the MMP-9 molecular model 1GKC and HDL model 3K2S. The crystal structure of HDL and MMP-9 were taken from protein data bank(PDB) database. The possibility of binding, precise location of binding site and the mode of binding between HDL and MMP-9 were carried out using an automated docking software (ZDOCK protocol). The ZDOCK protocol was used for docking HDL to MMP-9 which performs a six dimensional search between the two molecules(Wiehe et al., 2005). In the initial stage of ZDOCK ,MMP-9 and HDL were treated as rigid bodies and the docking were performed in the PDB site record. An evenly distributed rotational search resulted in 2000 poses. The near native structures obtained in the initial stage were refined and re-ranked using RDOCK. The best ranked pose was then used for the study. The results [Fig 21] indicated that pose 13 has the best Z rank score - 68.973 compared to others, predicting a greater possibility of binding of MMP9 with HDL. Further examination of this interaction revealed that MMP9 interaction with HDL involved 10 hydrogen bonds thereby showing a strong interaction between the two molecules.

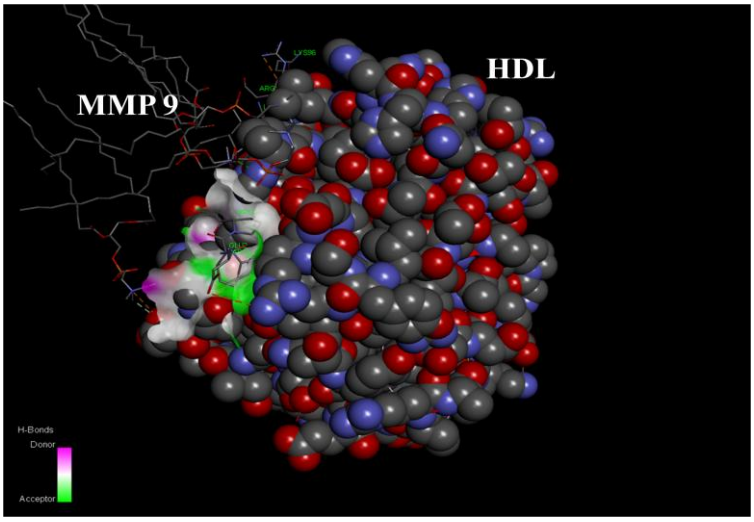


Figure 21: Docked structure showing interaction between HDL and MMP 9. *For protein –protein docking studies , ZDOCK protocol was used for docking HDL to MMP9. Docking result showing interacting residues between HDL active site & MMP9 (represented as CPK – Ball like structure) .*

4.6. Influence of Particulate matter associated with air pollution (polycyclic aromatic hydrocarbons) on HDL functionality

Apart from MMP-9(an intrinsic factor), environmental factors including particulate matter (PM) in air pollution can modify HDL functionality (Ramanathan et al., 2015). Epidemiological studies show that exposure to increased levels of air pollutants are positively associated with cardiovascular mortality (Pope et al., 2004, Brook. R.D etal, 2010). Air pollution is a heterogeneous mixture of PM and gaseseous components. Exposure to ambient $PM_{2.5}$ (PM <2.5 μm) has been reported to be associated with the rate of carotid intima-media thickness progression over time(Kunzli et al., 2010, Adar et al., 2013). Brief exposures of humans to fine concentrated ambient particle ($PM_{2.5}$) has been recently reported to elicit adverse effects on HDL functionality (Ramanathan et al., 2015). The present study investigated whether exposure of isolated HDL to polycyclic aromatic

hydrocarbons(PAH)[one of the most widespread organic pollutant in diesel exhaust, known for its adverse health effects], could influence functional alteration in HDL. To examine the effects of PAH components [eg.1,4 naphthaquinone and 9,10 phenanthraquinone] on HDL functional alteration,an aliquot of HDL was treated with 1,4 naphthaquinone and 9,10 phenanthraquinone at two different concentrations 0.1 and 1µM/ml for 1h[Fig 22] , and subjected to DCFH assay. Both PAH at 1µM/ml were found to induce significant impairement in HDL function as evidenced by increased fluorescence intensity.

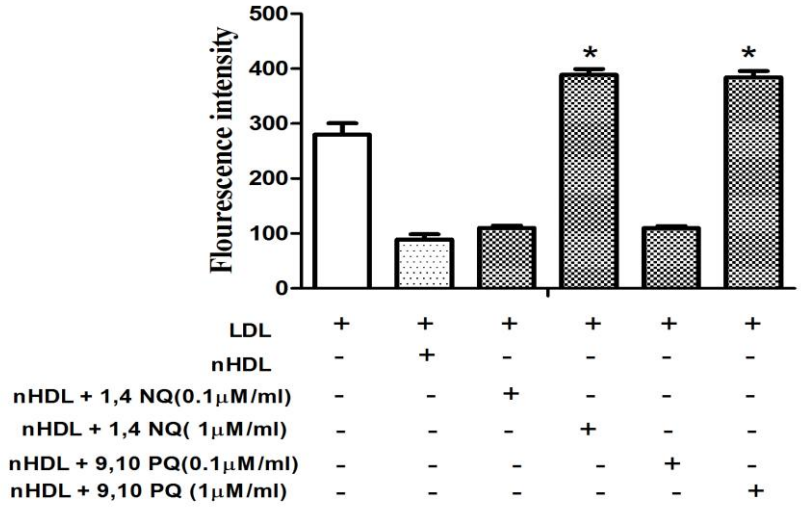


Figure 22: Effect of PAH components on HDL functionality. *An aliquot of HDL(350µg HDL-C/ml) was treated with 1,4 Naphthaquinone or 9,10 Phenanthraquinone at two concentrations 0.1 and 1.0 µM/ml for 1 hr and assayed for HDL functionality as detailed in methodology section.*

4.7. Investigation of the effect of dysfunctional HDL on human monocyte-derived- macrophage function relevant to atherosclerosis

Atherosclerosis can be considered as a chronic vascular inflammatory disease(Zernecke and Weber, 2010). HDL particles possess many antiatherosclerotic

properties including promotion of reverse cholesterol transport (RCT), antioxidative, antiinflammatory, antiapoptotic, antithrombotic, anti-infectious and vasodilatory effects(Eren et al., 2014). However, all HDL are functionally not equivalent. It can undergo pronounced compositional and functional modification in subjects under certain conditions associated with acute-phase response and inflammation(Hima Bindu et al., 2011). The present study has demonstrated that HDL from patients with established coronary artery disease, exhibits dysfunctionality in terms of its antioxidant ability to protect against LDL oxidation in vitro, and it promotes pro-oxidant effects in human monocytes(Sini etal, 2013). Further HDL characterization revealed an exclusive association of matrix metalloproteinase-9[MMP-9] with dysfunctional HDL and demonstrated its proinflammatory property (Sini etal 2014).

HDL normally plays a cardioprotective role by promoting RCT and modulating inflammation. In the RCT pathway, HDL mediates the transport of cholesterol from peripheral tissues, including arterial macrophages, to the liver for excretion as bile acids and free cholesterol(Tall,2015). RCT involves many steps, including the removal of cholesterol from cells through ABCA1, ABCG1, and SR-BI(Annema et al., 2010).The present study sought to explore the influence of dysfunctional HDL on lipid homeostasis, mainly cholesterol influx/efflux in human monocyte-derived-macrophages. To examine the cellular effects only 6 randomly selected samples each from healthy subjects and CAD patients were used.

4.7.1. Dysfunctional HDL and cholesterol homeostasis in monocyte-macrophages

4.7.1.1. Human macrophage culture

Human blood monocytes were isolated and cultured under standard conditions. Cells were grown in the culture medium supplemented with 10% autologous human serum for 8 days. The medium was replaced every two days. The morphology of the cells was monitored under microscope and the immunocytochemical analysis showed the predominanace of CD68 expression [Fig.23]. Trypan blue exclusion test was employed to assess the viability of cells and was found to be greater than 95%.

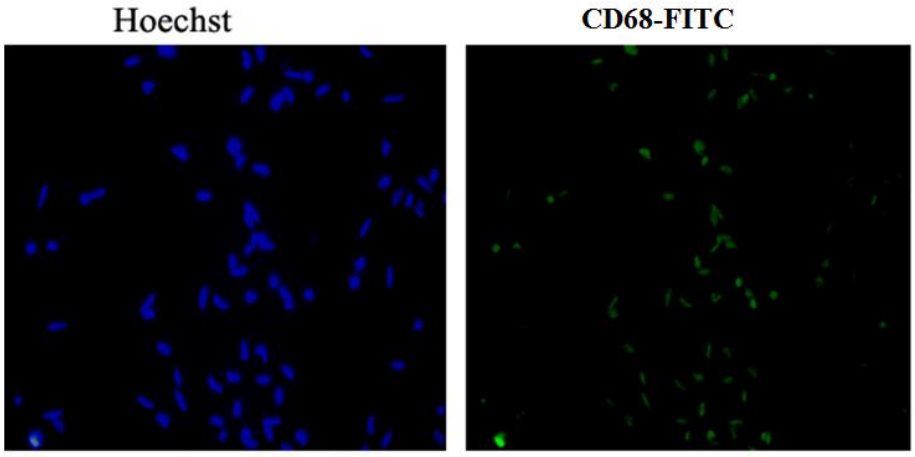


Figure 23: CD68+ cells(macrophages). *Monocytes($1x10^6$) after 8 days of culture were stained with FITC labeled CD68 marker and fluorescent image was taken with Hoechst as counter stain using (blue and green filter) under fluorescent microscope at 20 X magnification(IX 51 inverted basic microscope, Olympus, Japan). A- Hoechst staining of cells. B- corresponding FITC image showing CD68+ cells.*

4.7.1.2. Cell treatment

Macrophages maintained in culture as described above, were serum-starved overnight and then incubated with medium containing PBS alone, functional HDL

(nHDL) or dysfunctional/ pro-inflammatory HDL(piHDL) from CAD patients (antioxidant capacity assessed based on the functional assay of HDL) at a concentration of 50 μg protein/ml for 24 hours. Cell culture supernatants were collected and cells were dislodged by 3-mM EDTA treatment.

4.7.1.3. piHDL induces oxidative stress, lipid accumulation and foam cell formation in human monocyte-derived- macrophages

The ability of HDL to promote cholesterol efflux from macrophages is an important anti-atherogenic mechanism. To investigate the functional properties of piHDL in regard to atheroma formation, its effect on macrophage ROS generation and foam cell formation was studied. Human peripheral blood monocyte-derived macrophages were used since macrophages are primary target cells for atherogenesis. It was observed that piHDL treatment for 24 h significantly enhanced the production of intracellular ROS in macrophages as noted by DCFH fluorescence[Fig.24A &-B], indicating its pro-oxidant property, while nHDL did not stimulate ROS formation. In addition, marked difference in the accumulation of intracellular lipids, between cells treated with nHDL and piHDL was observed. As shown in Fig.24C and -D , exposure of macrophages to piHDL resulted marked increase in cellular total cholesterol content, with concomitant increase in foam cells as evidenced by the presence of Oil red O - stained lipid droplets.

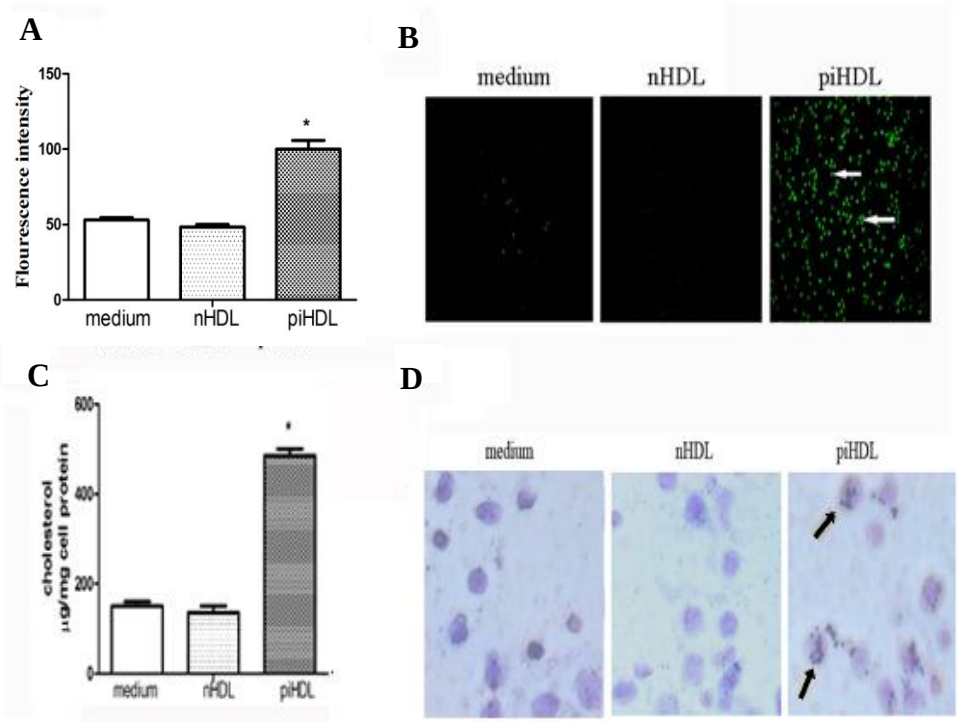


Figure 24: Dysfunctional HDL induces ROS production and lipid accumulation in human monocyte-derived macrophages. *Monocytes (1×10^6/ ml) were cultured for 8 days for differentiation into macrophages and treated with medium alone, nHDL or piHDL at 50µg/ml isolated from different individuals for 24h . Fig 24A) Intracellular ROS production measured as DCF fluorescence intensity. Fig 24B) DCF fluorescence visualized under fluorescent microscope at 20 X magnification(IX 51 inverted basic microscope, Olympus, Japan). Fig 24C) Cellular total cholesterol content quantitated using enzyme assay kit. Fig 24D) Macrophages stained with Oil red O for neutral lipids, viewed under inverted-microscope at 20X magnification. Arrows indicating Oil red O-positive lipid droplets. Values are expressed as mean ±SD of six experiments, '*'p <0.001 vs nHDL*

4.7.1.4. Assessment of expression of LOX1, ABCG1, SR-B1 and CD36 in macrophages

The accumulation of intracellular lipids is the result of an imbalance between the influx and efflux of lipids. This study demonstrates that piHDL could stimulate cholesterol influx and lipid accumulation in macrophages, favouring pro-atherogenic

mechanism. Here, the study sought to investigate the possible involvement of important receptors such as SRB1 (that promotes the bidirectional flux of free cholesterol between macrophages and lipoproteins), CD36(a multi ligand scavenger receptor), LOX1 (a major receptor for oxidized-LDL)and ABCG1(cellular cholesterol exporter to HDL), in mediating cholesterol influx / efflux in response to piHDL. Using western blotting as shown in Fig 25-A & -B it was observed that exposure to piHDL profoundly upregulated CD36 expression and moderately enhanced LOX1 expression in macrophages compared to nHDL. In contrast, the expression levels of ABCG1 and SRB1 were found to be suppressed by piHDL. The results observed provide clear evidence for the differential regulation of CD36 and SRB1 in macrophages by the two forms of HDL. While nHDL showed an inhibitory effect on CD36 activation, piHDL showed upregulation of CD36 expression in macrophages. Similarly, when nHDL strongly activated macrophage SRB1, piHDL markedly inhibited SRB1 expression. These differences in CD36 and SRB1 expressions appear to be important contributing factors for enhanced lipid uptake in macrophages. LOX1 does not appear to have any significant role, as its expression is marginally enhanced by piHDL.

Next objective was to determine the effect of piHDL on CD36 mRNA expression using qRT-PCR analysis. Total RNA was extracted and used to synthesis cDNA, which was then amplified with or without SYBR Green for detecting CD36 expression. As shown in Fig.25C piHDL enhanced CD36 mRNA expression significantly in macrophages compared to nHDL. These data indicate that both the protein and mRNA expression levels of scavenger receptor CD36 were upregulated

by piHDL. Taken together, this findings demonstrate that reduced expression of ABCG1 and over expression of CD36 in response to piHDL impair the ability of macrophages to effectively regulate cholesterol homeostasis resulting in cholesterol accumulation.

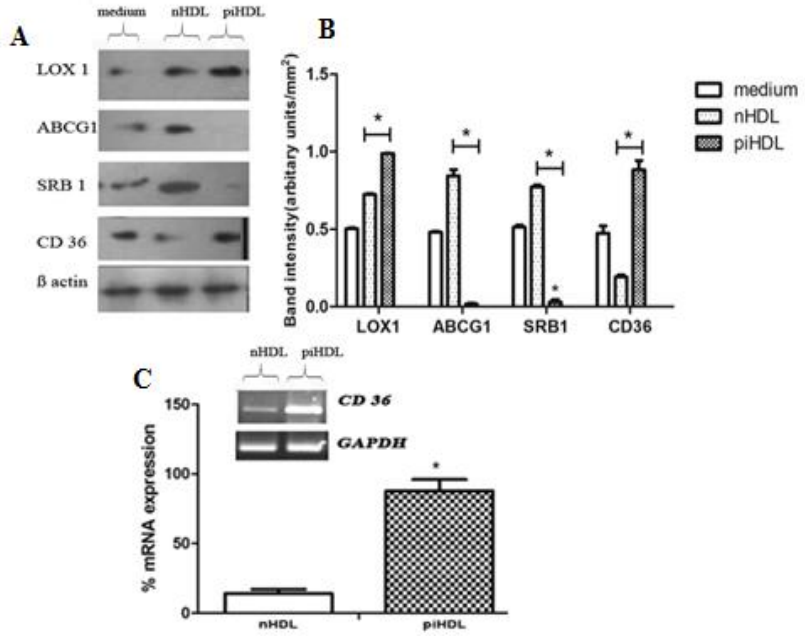


Figure 25: Macrophage receptor expression following exposure to HDL.*Monocyte-derived macrophages (1×10^6/ ml) were treated with medium alone, nHDL or piHDL (50µg/ml) isolated from different individuals for 24 h and examined for different protein expression levels as detailed in methodology using β-actin as loading control. (Fig 25A) Western blot of LOX1, ABCG1, SRB1 and CD36 expression. Protein bands were detected using the chemiluminescent ECL detection system and image was scanned using image scanner. Fig 25B. Band intensity for LOX1, ABCG1, SRB1 and CD36 using densitometry [ImageJ software,NIH) . Data is presented as mean±SD from six independent experiments. '*'p <0.05 vs nHDL. (Fig 25C) qRT- PCR analysis of CD36 mRNA expression with GAPDH as house keeping gene. Band intensity of CD36 mRNA using densitometry (ImageJ software,NIH). Values are presents as mean ±SD of three experiments. '*'p <0.001 vs nHDL.*

4.7.1.5. piHDL stimulates ERK1/2 MAPK and NFkB expression

It is well recognized that atherosclerosis involves an ongoing inflammatory response. Previous study indicates that dysfunctional HDL could promote inflammatory response in macrophages as evidenced by increased production of TNF-α and MMP-9(Sini etal, 2013). Here the effect of piHDL on regulation of MAPKs as well as the transcription factor- NFkB in macrophages was examined. Western blotting was employed to assess the protein expression levels of p38, JNK and ERK 1/2 MAPKs(Fig.26A & -B) and NFkB(p65 subunit) (Fig.26C &-D). In contrast to nHDL, piHDL treatment resulted in a profound increase in the expression of ERK 1/2 in macrophages with no evidence for the activation of JNK and p38 MAPKs. A concomitant increase was also observed in NFkB expression in response to piHDL, indicating the activation of inflammatory pathways.

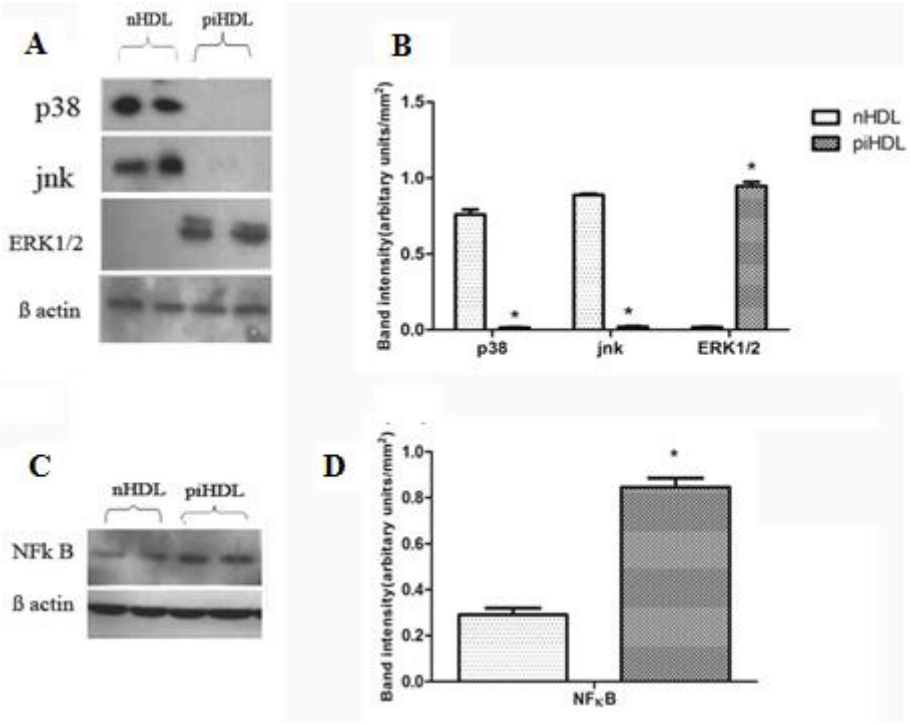


Figure 26: Influence of piHDL and nHDL on MAPKs and NFkB expression. *Monocyte-derived-macrophages(1×10^6/ml)were treated with nHDL or piHDL (50µg/ml) isolated from different individuals for 24h and subjected to western blot analysis as detailed in methodology section using β-actin as loading control. Western blot of p38, JNK, ERK1/2 -MAPK expression (Fig.26A) and NFkB(p65) expression(Fig.26C). Band intensity of p38, JNK, ERK1/2 -MAPK (Fig.26B) and NFkB(26D) using ImageJ software,(NIH). Data is presented as mean±SD from six experiments, '*'p <0.01*

4.7.1.6. piHDL induces CD36-dependent activation of ERK1/2 in macrophages

It was observed that both CD36 and ERK-MAPK pathways are activated in macrophages in response to piHDL. CD36, a multifunctional protein, has been reported to play a critical role in activating MAPK pathway(Silverstein et al., 2010) in addition to lipid homeostasis. In view of this, the possibility that CD36 could be playing a pivotal role in ERK activation in macrophages was explored. To validate the specific role of CD36 in the regulation of piHDL-mediated activation of ERK1/2

and associated cellular responses, pharmacological inhibition of CD36 was carried out using SSO [sulfosuccinimidyl oleate] before exposure to piHDL. It was observed that SSO essentially inhibited not only lipid accumulation but also inhibited the expression of ERK1/2MAPK [Fig.27A], NFkB[Fig.27B] , release of TNF-α[Fig27D], and MMP-9[Fig27E] . Fig 27C shows that inhibition of CD36 led to remarkable reduction in total cellular cholesterol content. In addition, CD36 blocking by SSO enhanced IL-10 release[Fig.27D] in macrophages. These findings using SSO showed that ERK1/2 activation was mediated by CD36. Next study evaluated the role of ERK-MAPK signaling, using PD98059(an inhibitor of ERK-MAPK) in piHDL-mediated CD36 expression in macrophages. It was observed that ERK1/2-MAPK inhibition remarkably blocked piHDL-induced CD36 expression at both protein[Fig.27F] and mRNA levels[Fig.27G] and subsequent lipid accumulation in macrophages[Fig.27H]. A concomitant reduction in NFkB expression was also observed. However, blockade of ERK-MAPK did not cause any obvious change in LOX1 expression in response to piHDL or nHDL treatment . The results collectively demonstrated that ERK-MAPK activation is necessary for piHDL-stimulated CD 36 expression.

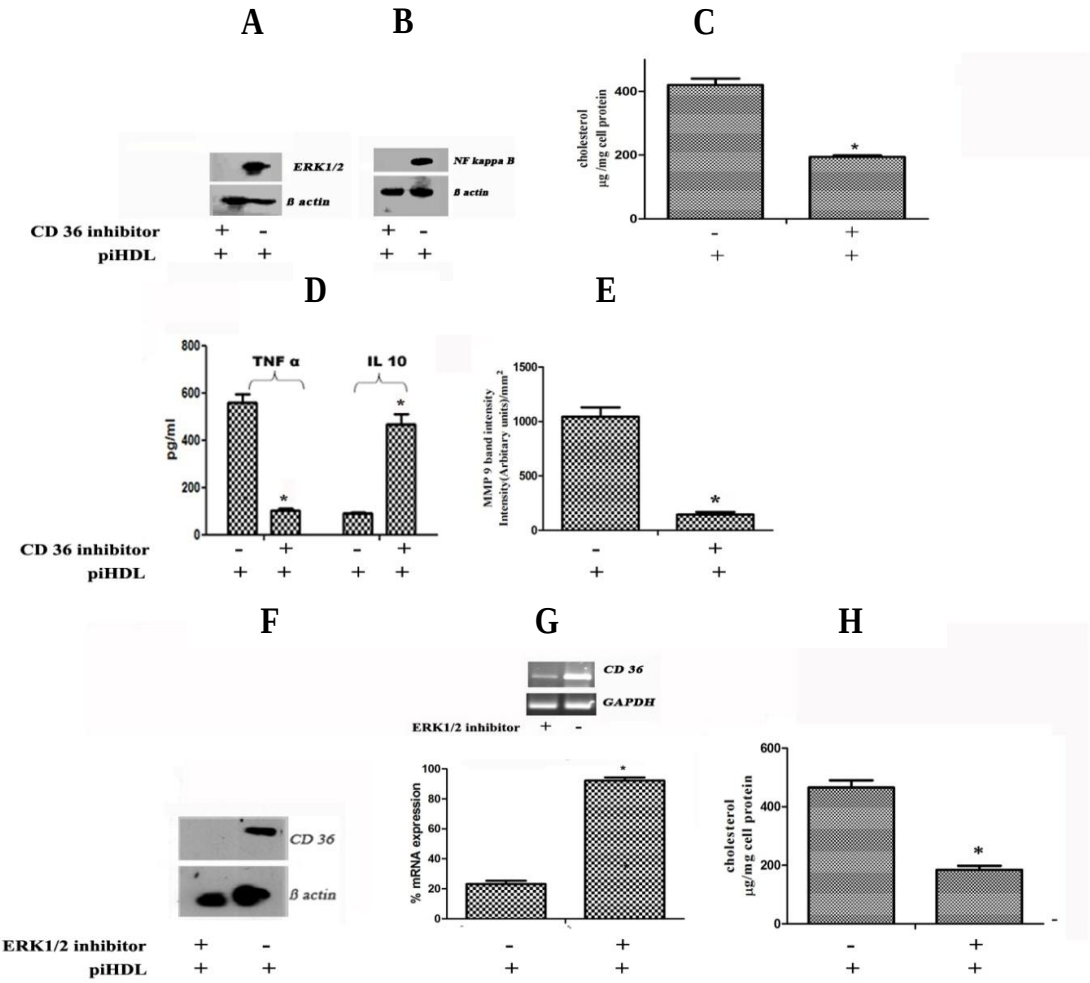


Figure 27: Cross- talk between CD 36 and ERK1/2 expression in macrophages following exposure to piHDL. *Monocyte-derived macrophages (1×10^6/ ml) were pretreated with CD36 inhibitor, SSO (10µM) for 2 h and then treated with piHDL (50 µg /ml) isolated from different individuals for 24h and examined for expression of (27A) ERK1/2 and (27B) NFkB by western blot, (27C) cellular total cholesterol content,(27D) release of cytokine - TNF-α& IL-10, and (27E)MMP-9 activity. In another set of experiment, macrophages were pretreated with ERK1/2 inhibitor, [pd98059(10µM)] for 2 h and treated with piHDL (50 µg/ml) for 24 h and then examined for expression of (27F) CD36 protein by western blot, (27G) CD36mRNA by RT- PCR with GAPDH as loading control and cellular total cholesterol content(24H). All data are expressed as mean ±SD from six experiments. '*'p <0.001 vs nHDL.*

4.7.1.7. piHDL activates PPAR-γ and Nrf2 in macrophages

CD36 expression on macrophages is mainly controlled by the nuclear receptor PPARγ(Olagnier et al., 2011) . Nrf2, is also shown to play an important role in the regulation of CD36 expression (Ishii et al., 2004). Therefore, the next study examined PPARγ and Nrf2 expression in macrophages after treatment with piHDL for 24h. Results obtained by western blotting showed that piHDL treatment moderately enhanced activation of both PPARγ and Nrf2 in macrophages [Fig 28A & -B].

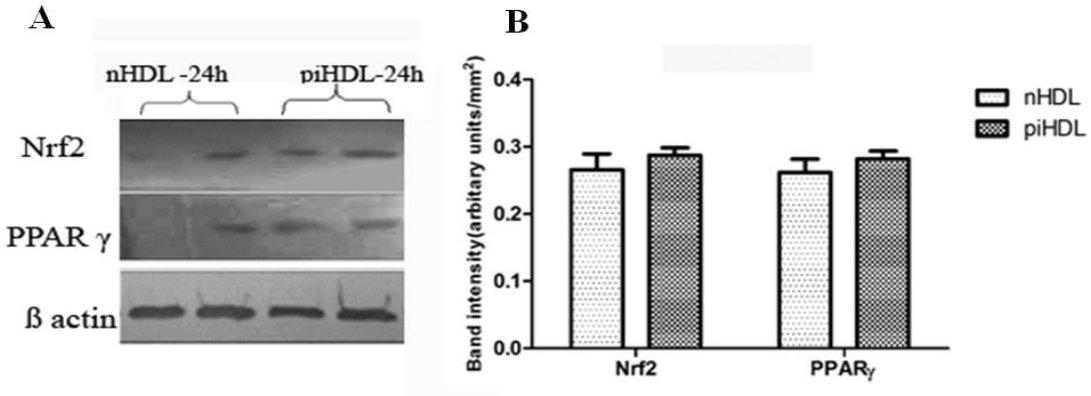


Figure 28: Influence of piHDL on expression of PPAR -γ and Nrf2 in macrophages. *Monocyte-derived- macrophages (1×10^6/ml) were treated with piHDL(50µg/ml) isolated from different individuals for 24h and examined for protein expression of PPAR -γ and Nrf2 by western blot analysis (fig 28A) with β actin as loading control. (Fig 28B).Mean band intensity ±SD from six experiments(ImageJ software, NIH).*

In conclusion the present study demonstrates that HDL from CAD subjects, in contrast to HDL from healthy subjects, promotes lipid accumulation leading to foam cell formation in human monocyte-derived-macrophages. The observed pro-atherognic effect of piHDL is mainly due to the upregulation of

macrophage scavenger receptor CD36. Furthermore the present study provides evidence for the activation of an intracellular signaling pathway, CD36-ERK/MAPK, in response to piHDL interaction with macrophages, thereby suggesting a molecular link that can promote the risk of CAD in subjects having dysfunctional/pro-inflammatory HDL.

4.8. Dysfunctional HDL and macrophage apoptosis

HDL is considered to have a critical role in macrophage apoptosis because of its anti apoptotic activity. Previous work demonstrates that dysfunctional HDL, in contrast to functional HDL, induces lipid uptake from piHDL leading to foam cell formation in human monocyte-derived-macrophages. The net effect of these changes induced by dysfunctional HDL has all of the pro-atherogenic characteristics that increase the inflammatory response, oxidative stress and lipid accumulation. These proatherogenic effects may also promote macrophage apoptosis. Apoptosis, a process of programmed cell death, is fundamental to normal development and maintenance of tissue homeostasis. However, macrophage apoptosis is also a key regulator in the development of atherosclerotic lesions(Littlewood and Bennett, 2003). The present study was to investigate the influence of pronflammatory HDL on macrophage apoptosis.

4.8.1. Dysfunctional HDL induces oxidative stress in human monocyte-derived macrophages

Oxidative stress represents an imbalance of redox signalling in the cells. To investigate whether dysfunctional HDL induced any oxidative stress, human macrophages were treated separately with native functional HDL from healthy

subjects or dysfunctional HDL from CAD patients at a concentration of 50µg/ml for different time intervals and the intracellular ROS generated were quantitated based on DCFH fluorescence [Fig 29]. Exposure of macrophages with dysfunctional HDL induced a steep and rapid ROS release at 5 min compared to nHDL[p <0.001], and subsequently the level declined nearly by half at 1 h and maintained that level up to 24h [p<0.001]. In contrast, ROS-production of cells, that were treated with medium[PBS] or nHDL remained at a basal level up to 24 h with short initial [at 5 min] ROS increase[p <0.05]. The small increase in fluorescence observed in control cells with and without added nHDL could be due to the presence of intracellular ROS continuously generated as part of cellular metabolism. The results showed that dysfunctional HDL treatment induced more oxidative stress in macrophages as noted by increased DCFH fluorescence than that of nHDL, indicating its pro-oxidant property[ROS fluorescence intensity at 24h-nHDL=59±5; piHDL=97±4 , '*'p <0.01].

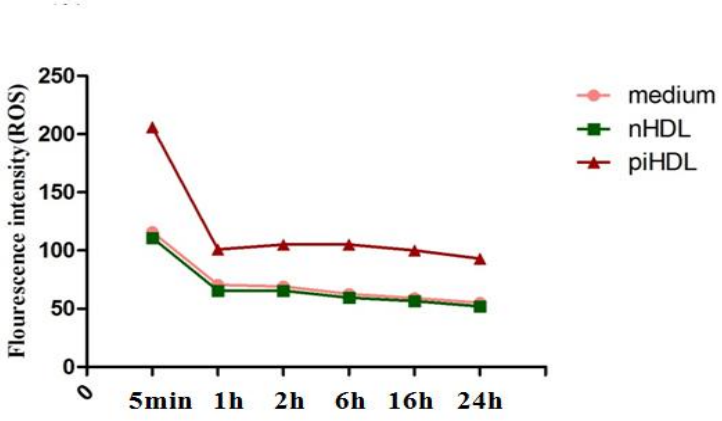


Figure 29: Time- dependent ROS production in macrophages.*Monocytes(1×10^6/ ml) were cultured for 8 days for differentiation into macrophages and treated with medium, nHDL or piHDL(50µg/ml) for different time intervals -5 min to 24 h. Values are the mean of two independent experiments.*

4.8.2. Sources of intracellular ROS stimulated by dysfunctional HDL

ROS have long been considered as by-products of metabolic processes and mediators of oxidative stress. ROS are generated by a variety of cellular sources including mitochondria, various oxido-reductases and metal-catalyzed oxidation of metabolites. Different enzymatic pathways may indeed contribute to the up-regulation of intracellular ROS production. To determine the major macrophage sources of ROS induced by piHDL, the activities of NADPH oxidase[NOX], xanthine oxidase(XO), cyclooxygenase (COX), and lipoxygenase (LOX) were assessed using enzyme inhibitors. Macrophages were incubated separately with specific inhibitors either apocynin [against NOX], allopurinol [against XO], indomethacin [against COX] or nordihydroguiaretic acid(NDGA) [against LOX] for one hour before treatment with HDL and the generation of intracellular ROS was measured using DCFH-DA. The results[Fig 30] showed that apocynin pre-treatment alone markedly inhibited ROS production in macrophages induced by piHDL indicating involvement of NADPH oxidase as the major source of intracellular ROS. Allopurinol, indomethacin or NDGA pre-treatment of macrophages showed no further decrease in ROS levels .

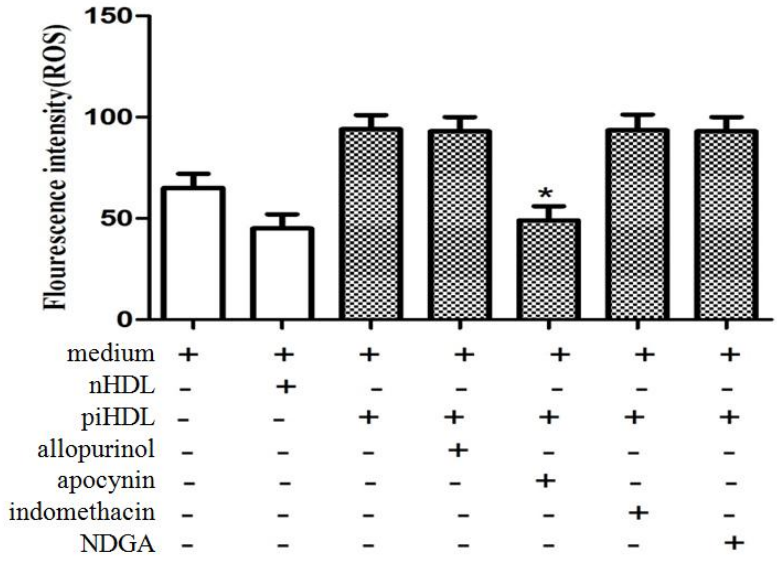


Figure 30: Sources of intracellular ROS induced by piHDL in macrophages. *Monocytes (1×10^6/ ml) were cultured for 8 days for differentiation into macrophages and treated with 10 μM of either allopurinol (XO inhibitor), apocynin (NOX inhibitor), indomethacin (COX inhibitor), or NDGA(LOX inhibitor), for 1h before treatment with nHDL or piHDL at 50μg/ml for 24h. The intracellular ROS generated were measured as DCFH fluorescence intensity. Values are the mean of six independent experiments ±SD, '*'p <0.05.*

4.8.3. Antioxidant enzyme status in human monocyte-derived macrophages exposed to piHDL

Cellular antioxidants can neutralize the deleterious effects of ROS.Next attempt was to study how piHDL regulates macrophage antioxidants, in comparison to nHDL, by examining the status of glutathione, activities of glutathione peroxidise, glutathione reductase,superoxide dismutase[SOD], and catalase. As shown in Table III, exposure of macrophages to piHDL resulted in a significant reduction in all the antioxidants when compared to that of nHDL.

Table III: Effect of piHDL on macrophage antioxidant status

Parameters (units/mg protein)	nHDL	piHDL
Reduced glutathione	24.6± 1.2	6.4 ± 0.32*
Glutathione peroxidise	22.8± 0.60	8.4 ± 0.56*
Glutathione reductase	23.6± 0.64	8.8 ± 0.60*
Superoxide dismutase	81.2± 0.89	53.2 ± 0.69*
Catalase	188.6±0.50	112.4 ± 0.46*

Values are the mean of six independent experiments ±SD, ''p <0.001*

4.8.4. Dysfunctional HDL induces lipid accumulation in human monocyte-derived macrophages

Formation of macrophage foam cells in the arterial intima play a critical role in the occurrence and development of atherosclerosis(Yu et al., 2013). Previous work demonstrated that dysfunctional HDL, in contrast to functional HDL, induced lipid uptake from piHDL leading to macrophage foam cell formation at 24h. Here the effect of dysfunctional HDL on macrophage cholesterol accumulation was examined at different time intervals [6h, 12 h and 24 h]. After treatment, cellular lipids were extracted and analysed for cholesterol content using enzyme assay kits. As shown in Fig 31, treatment of macrophages with piHDL resulted in maximum increase in cellular cholesterol content at 12h, which was about two fold higher compared to 6h. Macrophages exposed to piHDL for 24h showed almost similar cholesterol content as observed for 12h. In contrast, nHDL treatment maintained the basal cellular cholesterol content.

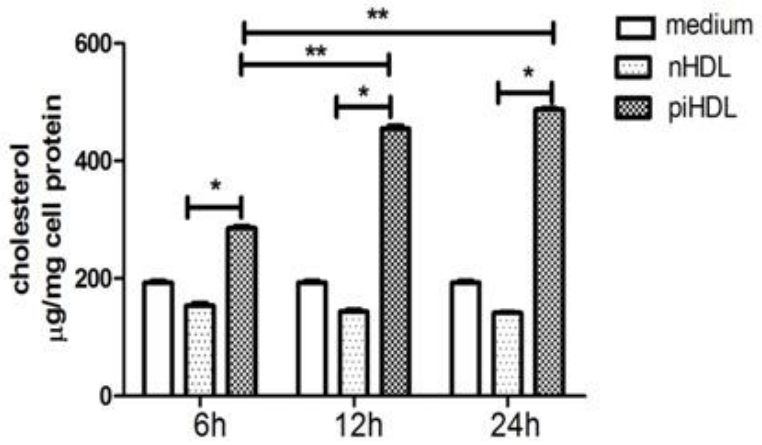


Figure 31: Time- dependent cellular cholesterol accumulation induced by piHDL.

Monocytes (1×10^6/ ml) were cultured for 8 days for differentiation into macrophages and treated with nHDL or piHDL at a concentration of 50µg/ml for 6h, 12h and 24h. Cellular total cholesterol content was quantitated using enzyme assay kit. Values are the mean of six independent experiments ±SD, [''p <0.01 nHDL vs piHDL, '*'p <0.001, piHDL at 6h vs piHDL at 12h/24h]*

4.8.5. Dysfunctional HDL induces cytotoxicity in human monocyte-derived macrophages

To examine the effect of piHDL on cytotoxicity, human macrophages were exposed to piHDL at different time intervals [6h, 12h,16h & 24 h].The cell viability rate was quantitated by MTT assay, which is based on the redox potential of NAD(P)H-dependent oxido-reductase in actively respiring cells to reduce MTT to its formasan. As shown in Fig 32A, piHDL induced cell death in a time-dependent manner. The cytotoxicity was found to be about 15% at 12h and more than 80% at 24h. Another set of experiment was conducted to study the cytotoxic effect of piHDL

in comparison to nHDL using Hoeschst-PI double staining. As shown in Fig 32B. piHDL induced more cell death as evidenced from remarkable increase in PI uptake in comparison to nHDL.

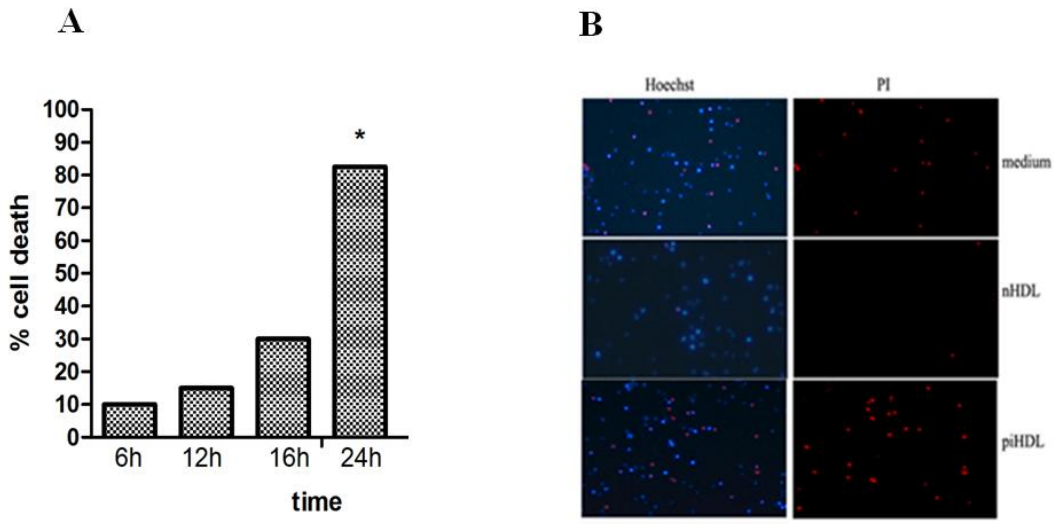


Figure 32: Dysfunctional HDL induced cell death in human macrophages. *Monocytes(1×10^6/ml) were cultured for 8 days for differentiation into macrophages and treated with piHDL(50µg/ml)and examined for cell viability using MTT assay(fig 32A). Values are the mean of six independent experiments ±SD, '*'p <0.001. Fig 32B, A representative micrograph of cells treated with medium alone, nHDL or piHDL at 50µg/ml for 24h and stained with Hoeschst-Propidium iodide double staining, obtained in an inverted-microscope (Olympus IX70 Barcelona, Spain) at 20 X magnification using appropriate filters.*

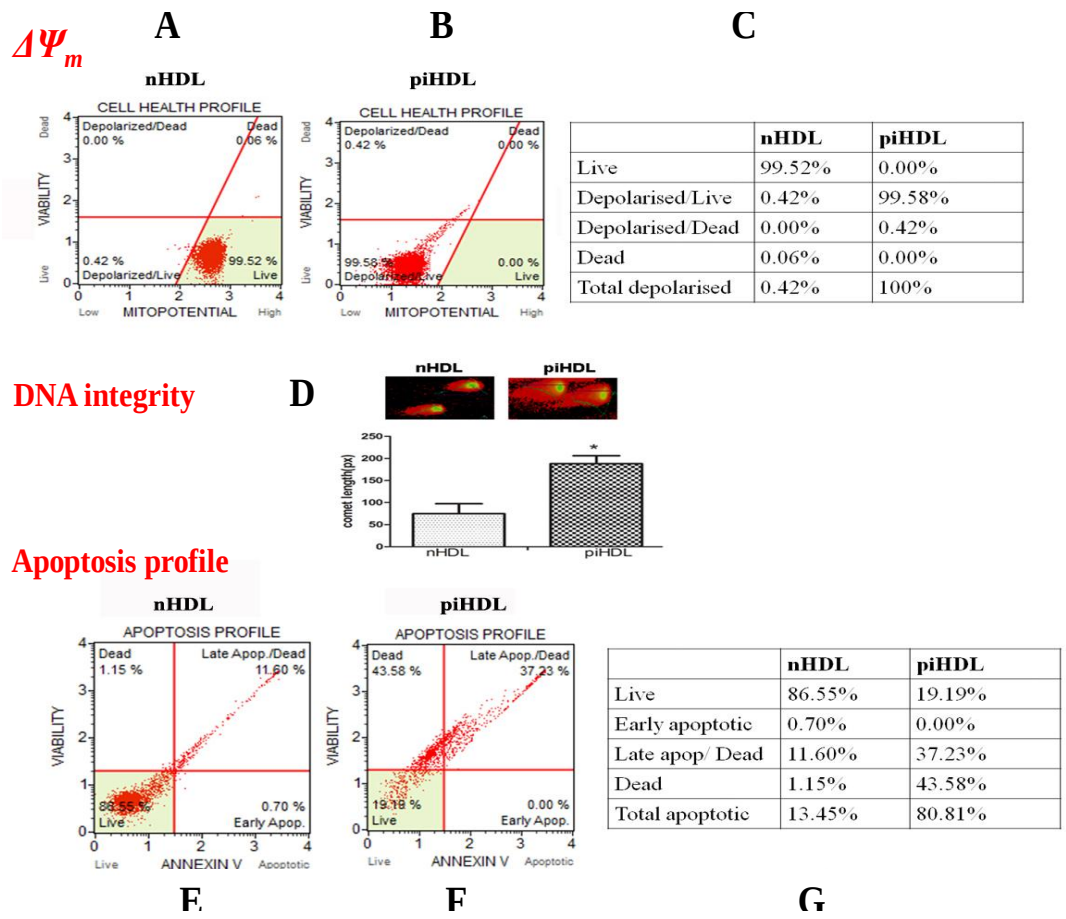
4.8.6. Dysfunctional HDL induces mitochondrial membrane potential ($\Delta\Psi_m$) alteration, DNA damage and apoptosis in human monocyte-derived macrophages

To examine the biochemical processes involved in marophage death due to piHDL, different assays including mitochondral membrane potential (Δφm) alteration using Muse potential assay kit and DNA damage were employed. A flourescent mitopotential dye that incorporate into mitochondria in a Δφm dependent

manner was used to detect changes in mitochondrial membrane potential of macrophages after treatment with piHDL. A representative flow cytometry data of $\Delta\varphi m$ is presented in Fig.33A,-B, &-C. As shown in figure, a remarkable loss in $\Delta\varphi m$ was observed, as evidenced by 100% depolarised cells, in macrophages treated wth piHDL.But nHDL treatment maintained essential membrane potential as indicated by reduced membrane depolarised cells[0.36±0.13,n=4].

Apoptosis was frequently associated with mitochondrial depolarization, resulting in increased numbers of depolarised cells. Comet assay [single-cell gel electrophoresis] was next employed to detect the DNA damage and the results are represented in Fig.33D. As shown in figure, nHDL treatment maintained the DNA integrity. In contrast, piHDL treated macropahges showed significant DNA damage as evidenced by increased comet length [length of DNA in tail].

Exposure to piHDL stimulates many events in macrophages including loss of cell viability, mitochondrial membrane depolarisation and DNA damage, which are characteristics of apoptosis. The loss of cell viability was further characterised by flow cytometric analysis based on phosphatidylserine [PS] externalization, using FITC-labelled Annexin V [a phospholipid-binding protein]. As shown in figure fig 33E,-F &-G, piHDL caused significant cell death[cell death more than 80% [nHDL= 9.6±4 %, piHDL= 78±3 %, $p<0.001$, n=4] associated with PS exposure on the plasma membrane, indicating apoptosis. This loss in viable cells is in relatively good agreement with cell viability assay using MTT, depicting the pro-apoptotic potential of piHDL. In contrast, macrophages treated with nHDL for 24 h showed more than 88% of viable cells.



	nHDL	piHDL
Live	99.52%	0.00%
Depolarised/Live	0.42%	99.58%
Depolarised/Dead	0.00%	0.42%
Dead	0.06%	0.00%
Total depolarised	0.42%	100%

	nHDL	piHDL
Live	86.55%	19.19%
Early apoptotic	0.70%	0.00%
Late apop/ Dead	11.60%	37.23%
Dead	1.15%	43.58%
Total apoptotic	13.45%	80.81%

Figure 33: Mitochondrial membrane potential ($\Delta\Psi_m$)alteration,DNA damage and apoptosis in macrophages in response to piHDL. *Monocytes (1×10^6/ml) were cultured for 8 days for differentiation into macrophages and treated with nHDL or piHDL at 50µg/ml for 24h and subjected to flow cytometry analysis and comet assay.Flow cytometry analysis was carried out to examine $\Delta\Psi_m$ alteration using Muse mitopotential Assay kit as described in methodology section. Fig 33A, & -B. $\Delta\Psi_m$ alteration in macrophages in response to nHDL or piHDL and 33C. corresponding table showing the data of depolarised cells.Fig 33D.Extent of DNA damage in macrophages in response to piHDL was examined using Comet assay. The ethidium bromide stained DNA was visualized as 'comets' with a fluorescent microscope [Olympus CKX 41) at 40x magnification.The captured comet images were analyzed by Tritek comet scoring software. Representative comet images[DNA damage] of macrophages exposed to nHDL and piHDL and corresponding histogram[n=4, p<0.01]. Flow cytometric analysis for apoptosis was measured using annexin V kit in MUSE Flow Cytometer (Fig 33E & F) & Corresponding table(33G) showing the data of apoptotic cells. A representative picture from one of four experiments is shown.(The work was carried out at Biogenix research centre,Trivandrum).*

4.8.7. PARP [poly(ADP-ribose) polymerase] cleavage in macrophages treated with piHDL

A large number of apoptotic cells were detected by flow cytometric analysis in macrophages treated with piHDL.To support this finding western blot analysis for cleaved PARP [poly(ADP-ribose) polymerase] was carried out. PARP, an essential nuclear component for maintaining normal cell function and viability, is a target protein for caspase 3, that is cleaved during the apoptotic process. In this study macrophages were treated with piHDL for 24h, and subjected to western blot analysis for determining the expression of cleaved PARP using polyclonal antibodies for 116 kDa PARP and 86 kDa cleaved PARP. Immunoblot analysis (Fig 34) revealed that the cleaved fragment of PARP (86 kDa) was clearly detected at 24h after treatment with piHDL, indicating macrophage apoptosis.

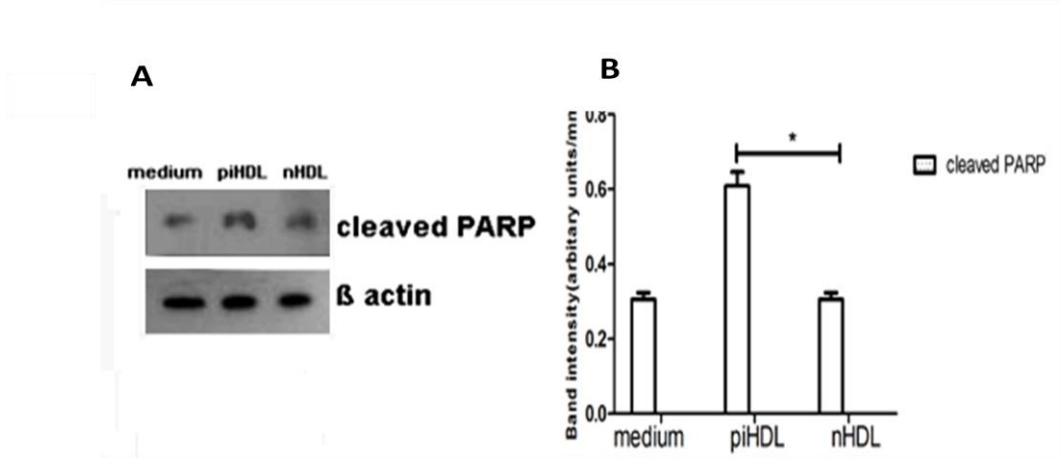


Figure 34: PARP cleavage in macrophages in response to piHDL.*Monocytes (1×10^6/ml) were cultured for 8 days for differentiation into macrophages and treated with nHDL or piHDL at 50µg/ml for 24h and subjected to western blot analysis for the expression of cleaved PARP[using cleaved PARP antibody] as detailed in methodology section. Fig 34A. A representative western blot using beta actin as loading control. Fig 34B. Band intensity of cleaved PARP using densitometry (ImageJ software,NIH). Values are the mean of six independent experiments ±SD, '*'p <0.001*

It was observed that apotosis induced by piHDL involves the cleavage of PARP[marker for apoptotic cell death] as evidenced by western blot analysis. Further the involvement of PARP in dysfunctional HDL induced apoptosis in macrophages was confirmed by benzamide, an inhibitor of PARP,(Fig 35) as evidenced by a remarkable reduction in apoptosis.

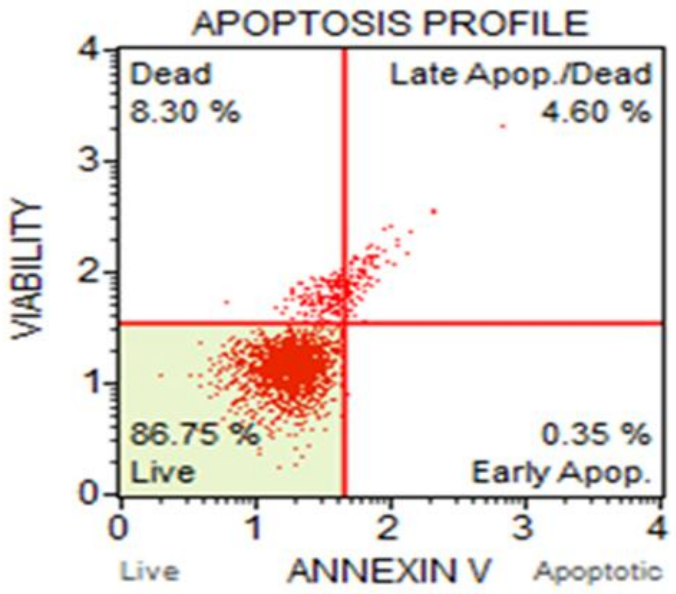


Figure 35: Benzamide inhibition of PARP cleavage in macrophage apoptosis. *Monocytes (1×10^6/ml) were cultured for 8 days for differentiation into macrophages and pretrated with PARP inhibitor, benzamide, for two hours and then treated with piHDL at 50µg/ml for 24h. Flow cytometric analysis for apoptosis using annexin V kit in MUSE Flow Cytometer.A representative profile from one of three experiments is shown. (The work was carried out at Biogenix research centre,Trivandrum).*

4.8.8. Dysfunctional HDL and macrophage autophagy

The engagement of autophagy in cell death regulation is observed under physiological and pathophysiological conditions(Martyniszyn et al., 2013). In order to examine the role of autophagy, western blot analysis was employed to determine the expression of LC3-1 and LC3-II[autophagosomal marker] in nHDL and piHDL treated macrophages, using anti-LC3-I (18kDa) and -LC3-II (16kDa) antibodies. As shown in Fig.36, a marked decrease in LC3-1 & -II protein expression was observed in macrophages after 24h treatment with piHDL, compared to nHDL, indicating a decrease in autophagic process in macrophages in response to piHDL exposure.

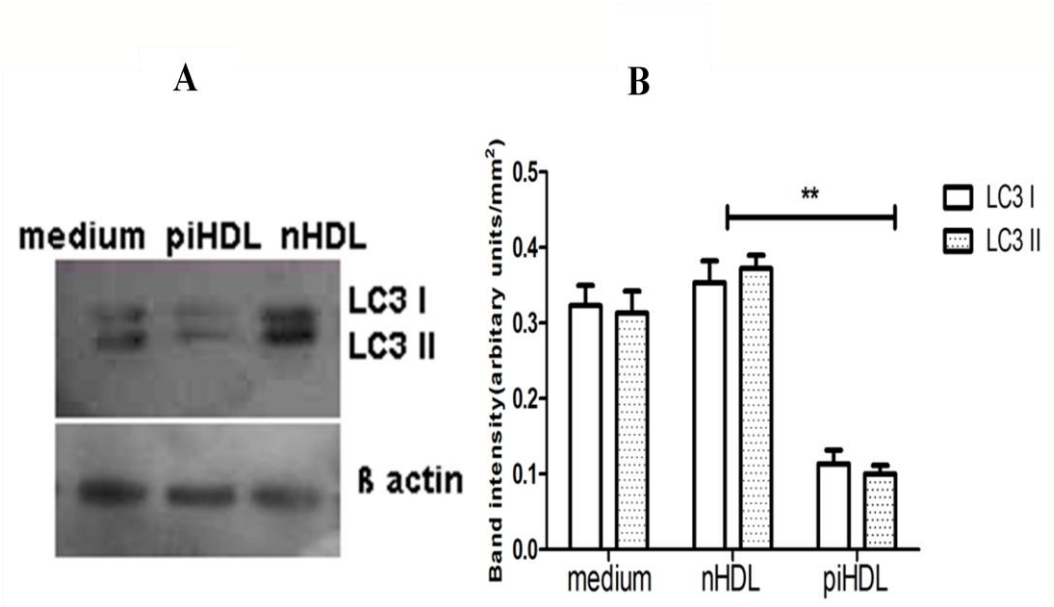


Figure 36: Autophagosomal marker-LC3-1 and LC3-II expression in macrophages. *Monocytes(1×10^6/ml) were cultured for 8 days for differentiation into macrophages and treated with nHDL or piHDL at 50µg/ml for 24h and subjected to western blot analysis for LC3 I and LC3 II as detailed in methodology section. Beta actin served as loading control. Fig 36A. A representative Western blot of LC3-I (18 kDa) and LC3-II (16 kDa) expression. Fig 36B. Band intensity of LC3 I and LC3 II using densitometry (ImageJ software,NIH). Values are the mean ±SD of six experiments. '*'p <0.001 vs nHDL.*

In conclusion the present study demonstrates that nHDL from healthy subjects maintains cellular cholesterol homeostasis, ROS generation, mitochondrial membrane potential, DNA integrity, autophagy and cell survival. In contrast, piHDL from CAD patients disturbs cellular metabolism resulting in cholesterol accumulation, excess generation of ROS, membrane depolarization, DNA damage and eventually apoptosis, thus demonstrating proatherogenic property. Macrophage apoptosis is an important feature in atherogenesis and is, in part, stimulated by dysfunctional HDL, thus providing new insights in our understanding of the atherogenic mechanisms.

DISCUSSION

5.1. Identification of the prevalence of functionally altered HDL in apparently healthy subjects and in patients with clinically diagnosed coronary artery disease

Atherosclerosis, the primary causative factor for CAD, is a complex inflammatory disease. An abundance of epidemiological evidence has documented an inverse association between HDL-C levels with the risk of CHD [(Gordon et al., 1977;Goldbourt et al., 1997;Lewington etal, 2007]. This relationship is supported by the potential anti-atherogenic properties of HDL, including cholesterol efflux from arterial macrophages, i.e. RCT activity, antioxidative, antiinflammatory, antithrombotic, vasodilatory and antiapoptotic effects(Besler etal, 2012; Rossensson etal ,2012, Negre-solvayra etal, 2006). Although HDL is atheroprotective it can become dysfunctional under inflammatory condition. In accordance with this, the present study identified functional impairement in HDL derived from subjects with clinically diagnosed CAD. This finding strongly supports the concept of functional alteration in HDL.

HDL possesses potent antioxidant/anti inflammatory property by virtue of its capacity to inhibit LDL oxidation, an early event in atherogenesis(Navab et al., 2011). In agreement to this, it was observed that HDL from majority of healthy subjects possesses remarkable antioxidant capacity to inhibit LDL oxidation. However, HDL from a few healthy subjects [~3% of the study group] and HDL from most of the CAD patients did not possess antioxidant activity and did not inhibit LDL oxidation, thus providing evidence for dysfunctional HDL. This finding is in consistent with the previous reports showing the prevalence of dysfunctional HDL in

CAD patients (Ansell et al., 2003, Dodani, 2008, Paneni et al., 2012). Dysfunctional HDL from CAD patients was found to be even proinflammatory, as it enhanced LDL oxidation. When the functional status of circulating HDL was assessed among healthy volunteers using larger sample size, it was observed that HDL functionality varied widely, irrespective of the level of HDL-C. This finding indicates that all HDL are functionally not equivalent.

HDL was found to be totally dysfunctional to inhibit LDL oxidation in subjects during systemic inflammation. Higher levels of hsCRP, a marker of systemic inflammation and gelatinase-B [MMP-9] were observed in subjects, both from control and CAD groups, presented with piHDL. A high level of CRP in the blood is a marker of any condition that causes inflammation. MMPs contribute both in the formation as well as in the destabilization of atherosclerotic plaque and also predict the cardiovascular risk in patients with CAD(Galis and Khatri, 2002). MMPs also involved in the homeostasis of the extracellular matrix(ECM)(Rodrigo et al., 2000). Serum levels of MMPs are very likely influenced by release of MMPs following degranulation of leukocytes and platelets and serve as a marker of pertinent intracellular protease activity. Alternatively elevated MMP9 concentration in serum might emanate from a circulating cell type associated with a pathological condition, suggesting that circulating leukocytes may be a source of the MMP 9 (Takeshita et al., 2001). In one study an association between inflammation and arterial stiffness has been reported (Kampus et al., 2006) and it suggests a role for gelatinase in large artery stiffening which is associated with an increased CV risk. As such, better understanding of the dysfunctional aspects of HDL metabolism and

gelatinase expression will become more important clinically and may also provide new strategies for its prevention/treatment.

The potent atheroprotective properties of HDL particle originate from their unique composition and structure(Eren et al., 2012). However, the underlying mechanism, which converts the normal HDL to a dysfunctional stage and its involvement in atherosclerosis development, are still unclear. In the present study, piHDL is characterized by decreased activity of PON-1, a HDL-associated antioxidant enzyme. Inaddition, piHDL shows enrichment of lipid peroxides, triglycerides and phospholipids. According to Kontush and Chapman, piHDL shows decreased levels of apoA-I and PON1(Kontush and Chapman, 2006).Some other studies have reported that dysfunctional HDL shows enrichment of oxidized phospholipids, triglycerides, myeloperoxidase, group IIA secretary phospholipase A2, ceruloplasmin, serum amyloid A and haptoglobin-hemoglobin complex (Asleh et al., 2008; Watanebe etal, 2010; Shah etal, 2013). The accumulated lipid peroxides and other oxidants in HDL can inhibit HDL-associated antioxidant enzymes, including PON-1, modify apoA1 and render the HDL particle unable to function as an effective antioxidant. In the dysfunctional stage HDL shows enrichment of TG, which is in agreement with Zago et al, who reported that the degree of HDL functional loss correlated with its triglyceride, but not cholesterol content(Zago et al., 2004). Thus, it is clear that HDL particles can vary in their atheroprotective capacity, and functionally deficient HDL may be involved with other atherogenic factors in the development of atherosclerosis. In support of this, an association between dysfunctionalilty in HDL and higher levels of serum TG, phospholipids, enhanced

systemic oxidative stress and inflammatory response was observed in few healthy subjects as well as in subjects with established CAD. These findings suggest that the functional loss in HDL might arise as an adaptive response to acute phase state, but when persistently expressed, this piHDL can induce chronic metabolic changes that drive further atherogenesis, eventually negatively impacting clinical outcomes.

Conventionally, HDL concentration is reported in terms of the cholesterol concentration in HDL and it cannot be considered as a surrogate marker of HDL functionality. HDL function has become an area of interest, as it is associated with critical antiatherosclerotic effects of HDL than with HDL-C. Since there is limited data reported regarding the rate of prevalence of piHDL, further study including larger sample size are needed to develop a database and to explore the factors causing dysfunctionality in HDL.

5.2. Association of matrix metalloproteinase-9(MMP-9) with dysfunctional HDL

HDL is a heterogeneous class of lipoprotein which differ by composition, shape, size and density(Gordon et al., 1977b). The antiatherogenic quality of HDL is defined by the functionality of HDL. The present study has demonstrated that the antiatherogenic property of HDL is impaired in subjects during inflammatory condition. It was also observed that serum gelatinases-MMP-2 and MMP-9, were significantly enhanced in subjects exhibiting piHDL. In accordance with previous reports the antiatherogenic functionality of HDL was found inversely associated with systemic inflammation (Eren et al., 2012, Kontush and Chapman, 2006).

In the current study, zymography and western blot analyses were employed to identify HDL-associated gelatinases and the data revealed that MMP-9 activity, but not MMP-2, was associated with piHDL. This observation remained consistent irrespective of isolation methods. Additionally, invitro studies demonstrated that when MMP-9 was added to HDL, MMP-9 could form complex with HDL and induce dysfunctionality in HDL. However, treatment with EDTA maintained HDL's antioxidant property. These invitro results provide evidence for a direct role of MMP-9 in HDL dysfunction. Human MMPs are multidomain enzymes and consist of catalytic- and Zn-binding domain, that together form the active site for proteolysis of substrates. This study could demonstrate that HDL-associated MMP-9 retains its gelatinolytic activity as evidenced from gelatin zymography data. In addition the association of MMP-9 does not induce significant proteolysis on apoA1, the major protein of HDL.These findings support the work of Lindstedt etal(Lindstedt et al., 1999), who reported that MMP-9 can form complex with HDL with no proteolysis on apoA1. How HDL-associated MMP-9 promotes the conversion of functional HDL to dysfunctional HDL is currently not well understood.

Previous proteomic studies of HDL provided evidence for the complexity of the HDL associated proteome, revealing HDL as a platform for distinct proteins involved in homeostasis, thrombosis, immune and complement systems(Karlsson et al., 2005).Watanabe et al using proteomic analysis have indicated that pro-inflammatory HDL contains a significantly altered proteome(Watanabe et al., 2012). Indeed proteomic studies described do not report the identification of MMP-9 in HDL, which may be due to technical problems associated with the methods of HDL

isolation, separation and/or analytical approach to individual protein detection from complex mixtures or proteolytic digests by MS/MS technology. This study aimed at identification of specific protein, i.e.MMP-9 in HDL, using immuno detection method, could reproducibly detect the association of MMP-9 with HDL. In agreement to the previous studies, the present finding i.e. HDL-associated MMP-9, implicates HDL as an active participant in the inflammatory response, carrying multiple proteins involved in inflammation. Generally MMP-9 is found at very low levels in the serum of healthy individuals, but substantially elevated levels are observed in the serum of those with systemic inflammation and acute coronary syndromes(Takeshita, 2001). MMPs are increasingly expressed in macrophage-rich areas of the atherosclerotic plaque, which might promote weakening of the fibrous cap and subsequent destabilization of atherosclerotic lesions. Since MMP9 plays an important role in plaque formation and destabilization, the formation of HDL-MMP-9 complex may have important clinical implications. Further investigations with a larger sample size will be needed to determine whether MMP 9 association with HDL can be used as relevant cardio vascular risk biomarker.

To my knowledge, this is the first report, which explored the novel association of MMP-9 with dysfunctional HDL and thus provides new insights into the atherogenic role of dysfunctional HDL. A decreased activity of PON-1 in the dysfunctional HDL compared to functional HDL, was also noted in this study. When monocyte/macrophage were treated with dysfunctional forms of HDL (HDL isolated from CAD patients and HDL treated with MMP-9), the release of TNF-α was found to be significantly higher and that of IL-10 was significantly lower as compared to

the response to functional HDL. These results suggest that dysfunctional HDL can acquire pro-inflammatory property, due to its content of altered proteome, lipids , its oxidation products and possibly, due to its association with the protease-MMP-9. The invitro findings of functional impairment in MMP-9 treated HDL, and subsequent stimulation of TNF-α production in monocytes/macrophages, support the importance of HDL-associated MMP-9. This finding is in agreement with the report of Yamamoto etal(Yamamoto et al., 2012) revealing that HDL of patients with end stage renal disease had increased macrophage cytokine response – in terms of TNF -α. Additionally, MMP-9 activity was found to be associated mainly with the HDL2 fraction, the more variable subclass of HDL, which reflects changes in HDL. Regarding HDL, circulating HDL2 subfraction was mainly found to protect from atherosclerosis(Lamarche et al., 1997). This finding suggests that the association of MMP-9 with HDL2 may negatively influence the protective role of HDL2 and enhance the risk for cardiovascular disease. These mechanisms are also relevant to atherosclerosis, and may help to explain the increased cardiovascular risk in subjects with dysfunctional HDL, despite a normal HDL-C level.

This is the first study to describe the previously unrecognised association of MMP-9 with dysfunctional HDL, using gelatin zymography and western blot analyses. These findings demonstrate that dysfunctional HDL carries MMP-9, that is secreted in circulation during inflammatory condition, with resultant loss of its anti-atherogenic property. The data presented here are highly significant as it provides new insights into the pro-inflammatory property of HDL.

5.3. Investigation of the effect of dysfunctional HDL on human monocyte-derived-macrophage function relevant to atherosclerosis

Macrophage foam cell formation in arterial wall is an essential, yet incompletely understood component in atherogenesis. Macrophages depend on reverse cholesterol transport mechanisms to remove excess cholesterol. Several critical lipoprotein receptors are involved in maintaining a balance between influx and efflux of lipids from macrophages(Reiss and Cronstein, 2012). HDL particles are capable of accepting cholesterol from macrophage foam cells and thereby maintaining net cholesterol balance. The pathways that regulate HDL-mediated macrophage cholesterol efflux involve cell membrane-bound transporters, including ABCA1, ABCG1 and SRB1(Rosenson et al., 2012). Evidence is now accumulating that HDL does not always have anti-atherogenic properties, that it may be dysfunctional under inflammatory conditions and even gain proatherogenic properties(Sini etal 2014). When treated with macrophages, piHDL, in contrast to nHDL, elicited cholesterol influx capacity of macrophages as noted by the over expression of CD36, that lead to lipid accumulation and foam cell formation. This defect in HDL functionality also resulted in suppressed expression of ABCG1, the major cholesterol transporter to mature HDL particle, and SRB1 that mediates cholesterol transfer to and from HDL, resulting insufficient activity of lipid efflux pathway. In such conditions, influx of cholesterol might exceed efflux, resulting in transformation of the macrophage into a foam cell phenotype. Under extreme-conditions of cholesterol accumulation by uncontrolled lipid accumulation via scavenger receptor (e.g. CD36), the cholesterol efflux pathway is suppressed and those of inflammatory signaling pathways are highly induced via activation of

CD36/ERK1/2-NFkB. The activation of inflammatory pathways can suppress LXR activity(Stirban et al., 2014) and its target genes causing decreased expression of ABCG1 and reduced cholesterol efflux. This may be general mechanism connecting ERK1/2-NFkB mediated inflammatory response to decreased RCT. These findings indicate that piHDL has pro-atherogenic property as it induces cholesterol accumulation in macrophages. The accumulation of lipid-laden macrophage foam cells in the intimal layer of artery is a characteristic feature of fatty streak, the earliest lesion of atherosclerosis.

Notably, an intracellular signaling pathway, CD36-ERK/MAPK, stimulated in macrophages in response to piHDL, was identified that is essential for lipid accumulation, foam cell formation as well as pro-inflammatory response. CD36 plays a major role in facilitating lipid uptake from piHDL. However it does not act alone, indeed it is at the point of interconnection of ERK/MAPK signaling pathway. In contrast to the influence of piHDL, nHDL reduced the uptake of lipids, down-regulated CD36 expression and activated ABCG1 and SRB1 on macrophages, thereby exhibiting its well recognized anti-atherogenic property. ABCA1 facilitates the efflux of phopholipids and cholesterol to lipid poor apoA-1 to generate nascent HDL particles, but ABCG1 facilitates efflux of cholesterol to mature HDL particle(Tall et al., 2008). Because mature HDL acts as an acceptor of ABCG1-effluxed cholesterol, the influence of HDL(mature HDL) on regulation of ABCG1 expression was examined. The relative role of ABCA1 was not assessed in the present study that forms a limitation.

CD36 is a multifunctional scavenger receptor(Silverstein and Febbraio, 2009). Because of its function in several signaling pathways, the next objective of this study was to explore whether CD36 could influence ERK1/2/MAPK activation. Blocking of CD36 with SSO, this study has established that ERK/MAPK is activated in a CD36-dependent manner in macrophages in response to piHDL. Furthermore, pre-treatment of macrophages with PD98059, a specific inhibitor of the ERK/MAPK, abolishes piHDL-mediated CD36 upregulation and lipid accumulation almost to the same extent as noted with SSO. PD98059 mediates its effect by preventing phosphorylation of ERK1/2[p44/p42 MAPK] by MEK1/2(Kim et al., 2006). These results indicate that CD36 and ERK/MAPK activation are collectively essential for piHDL-mediated lipid uptake in macrophages, which in turn activates NFkB and subsequent release of pro-inflammatory markers. The results suggest that CD36 may be acting as a signaling receptor and transmitting signals via ERK/MAPK. However, the precise mechanism that link CD36 and ERK/MAPK activation in response to piHDL is unknown at present. CD36 has been shown to localize at the plasma membrane and intracellular compartments(Feng et al., 2000). It is physically associated with src family kinases in membrane invaginations [caveole] that are enriched in a variety of signal transduction molecules(Huang et al., 1991) and the clustering of signaling proteins may result in more rapid cross talk and/or promote efficiency of signal transduction(El-Yassimi et al., 2008). It is likely that the plasma membrane localization of CD36 is important for the CD36-associated ERK1/2/MAPK activation in response to piHDL interaction with macrophage.

The cellular receptors for piHDL are poorly defined. Besler et al(Besler etal., 2012) have demonstrated that HDL from patients with CAD, i.e. dysfunctional-HDL, activates endothelial PKCβII, which in turn inhibits endothelial nitric oxide production through the activation of endothelial lectin-like oxidized LDL receptor-1(LOX-1). Both SR-B1 and LOX-1 on endothelial cells have been reported to bind hypochlorite-modified HDL(Marsche et al., 2001). LOX-1 is an endothelial receptor for oxidized low-density lipoprotein(oxLDL)(Chen et al., 2002). However, using human monocyte-derived-macrophages, the present study has demonstrated that CD36, a scavenger receptor for oxidized lipids, is upregulated abundantly in response to piHDL and it plays a major role in lipid uptake and associated cellular responses. In one study, macrophage CD36 activation has been observed to promote lipid uptake from copper- oxidized HDL, but not from native HDL or LDL(Thorne et al., 2007). The observed pro-atherogenic effects of piHDL are mainly depended on macrophage scavenger receptor CD36. LOX1 does not appear to have any significant role, as its expression is marginally enhanced by piHDL. In addition, the effects of both forms of HDL on LOX1 is found not to be associated with the activation of ERK/MAPK as evidenced from studies blocking ERK/MAPK pathway[data not shown]. Further, HDL from CAD[dysfunctional-HDL] has reduced PON1 activity and is rich in malondialdehyde (MDA)(Sini , 2013). As suggested by Besler et al(Besler et al., 2012), the marginal raise observed in macrophage LOX1 expression could be at least in part, due to the oxidized lipids in piHDL.

PPAR- γ and Nrf2 are known regulators of CD36 in macrophages(Olagnier et al., 2011, Nicholson, 2004). Increased expression of CD36 observed in this study,

could be due to PPAR-γ and Nrf2-the important regulators of CD36 expression, which are in fact expressed in macrophages upon exposure to piHDL. ROS activate Nrf2 and PPARγ ,that acts by a positive feedback mechanism resulting in the expression of transcription factors and/or antioxidant and pro-survival genes(Polvani et al., 2012). Although the present study provides evidence for piHDL-induced activation of PPAR-γ and Nrf2 along with CD36 and ERK/MAPK pathways in macrophages, little is known at present about the interplay between these pathways and how they coordinate to contribute to lipid accumulation.

The proatherogenic effects induced by piHDL are not fully understood. HDL is a heterogeneous class of lipoprotein which differs by composition, shape, size and density(Eren et al., 2014). It is known that dysfunctional HDL differs from normal HDL in its content of proteins and lipids. In the present study, the functionality as well as the chemical composition of both normal and dysfunctional HDL was analysed. Characterization of piHDL showed an enrichment of triglycerides, phospholipids, lipid peroxides, MMP-9 activity and decreased content of cholesterol and activity of paraoxonase-1, an anti-oxidant enzyme, compared to nHDL. piHDL contains a number of different oxidized compounds derived from both lipids and proteins, several acute phase proteins such as serum amyloid A(SAA), ceruloplasmin, myeloperoxidase(Watanabe etal,2012) and MMP-9(Sini etal 2014), that might interact with different cell surface receptors and/or proteins, alter cell membrane characteristics and promote activation of various intracellular pathways leading to proatherogenic responses. The present study for the first time to my knowledge provides evidence that macrophages when exposed to piHDL express

CD36 protein abundantly and upregulate ERK/MAPK signaling that facilitates lipid uptake from piHDL leading to foam cell formation. This study provides novel insights into understanding the pro-atherogenic mechanisms elicited by pro-inflammatory HDL from CAD subjects, and are highly relevant to the *in vivo* findings. Dodani et al have reported that the proinflammatory index of HDL[piHDL] is correlated with carotid intima-media thickness in South Asian immigrants(Dodani et al., 2014). It has also been reported that the capacity of HDL to promote cellular cholesterol efflux correlate more closely with carotid intima-media thickness than HDL-C concentration(Soran et al., 2012). A recent study shows that when oxidized at a specific site on apolipoprotein A1 [at Trp72] HDL becomes dysfunctional and proinflammatory(Huang et al., 2014) that it exerted a proinflammatory effect on endothelial cells as evidenced by increases in adhesion proteins and proinflammatory markers. Indeed, HDL isolated from atherosclerotic plaques have been reported to have impaired capacity to stimulate macrophage cholesterol efflux(Besler et al., 2011). While there is a growing body of literature describing the cardioprotective effects of HDL, we still have much to learn about HDL, a heterogeneous particle.

In conclusion the present study demonstrates that HDL from CAD subjects [piHDL], in contrast to HDL from healthy subjects [nHDL], induces lipid uptake from piHDL leading to foam cell formation in human monocyte-derived-macrophages. The observed pro-atherognic effect of piHDL is mainly due to the upregulation of macrophage scavenger receptor CD36. Furthermore the present study provides evidence for the activation of an intracellular signaling pathway, CD36-

ERK/MAPK, in response to piHDL interaction with macrophages, thereby demonstrating a possible mechanism for its proatherogenic potential.

5.4. Dysfunctional HDL and macrophage apoptosis

Macrophage apoptosis is an important feature of atherosclerotic plaque development in CAD. The processes and molecules that can stimulate the apoptotic program are diverse(Elmore, 2007). Although several factors are involved in macrophage apoptosis, HDL at physiologically relevant concentrations has a potent protective role against macrophage apoptosis(Terasaka et al., 2007). This study shows that dysfunctional HDL, in contrast to normal HDL, is able to induce apoptosis in human monocyte-derived macrophages as demonstrated by the results of cell viability, flow cytometry, DNA damage and PARP cleavage. Macrophage apoptosis occurs during all stages of atherosclerosis(Kockx et al., 1998), but the cellular mechanism for this process is largely unknown.

Monocyte-derived macrophages are central cells that accumulate cholesterol in atherosclerotic lesions(Kruth, 2001). In this study, the effect of piHDL on marophage lipid uptake was assessed at different time intervals up to 24h. When compared to nHDL, the intracellular cholesterol accumulation was peaked at 12h in macrophages cultured with piHDL, and maintained that level at 24h. Previous study identified the involvement of macrophage scavenger receptor -CD36, in lipid uptake from piHDL. In addition, the expression of SRB1 and ABCG1 that mediate the outflow of cholesterol was found decreased. The altered expression of macrophage receptors involved in cholesterol homeostasis can result an imbalance of influx and efflux of cholesterol and subsequent cholesterol accumulation.These results indicate

that piHDL, instead of regulating cholesterol homeostasis in macrophages as offered by nHDL, stimulates cholesterol accumulation and exhibiting proatherogenic property. In support of this finding, Westerterp et al(Westerterp et al., 2013) have shown that deficiency of ABCA1/G1 enhances lipid accumulation in macrophages, atherosclerosis and lesion inflammation. The HDL receptor ABCG1, has an essential role in promoting efflux of 7-ketosterol and related oxysterols from cells to HDL(Terasaka et al., 2007) and its deficiency results in accumulation of such oxysterols which have an important role in inducing ROS generation and apoptosis to variety of cell types(Kosmider et al., 2010). ABCG1 is also critical to the antiapoptotic effects of HDL in macrophages(Yvan-Charvet et al., 2010).

The next objective was to investigate the effects of nHDL and piHDL on macrophage survival/apoptosis. The study identified enhanced apoptotic cell death in macrophages after exposure to piHDL, by annexin V staining which is indicative of phosphatidylserine externalization. The study also examined the changes in ROS generation, mitochondrial membrane polarization/depolarization, DNA damage, expression of the protein markers associated with apoptosis (cleaved PARP), and autophagy [LC3-1 and LC3-II] in macrophages in order to understand the cellular events associated with cytoprotection /cytotoxicity. piHDL caused marked enhancement in intracellular ROS generation, mitochondrial membrane depolarization, [more than 99% of depolarized cells by flow cytometry] , DNA damage as revealed by increased length of comet and cleaved PARP by western blot,indicative of apoptosis. In contrast, similar concentration of nHDL could maintain the viability of most of the macrophages [viable cells=88.55%] at 24 h

treatment. Further, the data related with autophagic process demonstrate that nHDL treatment results in enhanced expression of LC3-I & LC3-II, [the autophagic proteins], in macrophages , reduced cleavage of PARP[apoptotic marker] and apoptosis. These effects, however, were lost when piHDL was used. The cleavage of PARP is considered to be one of the classical characteristics of apoptosis.

During apoptosis, 116 kDa PARP is cleaved by the activated caspase into 25 kDa and 86 kDa fragments(Chaitanya et al., 2010). PARP cleavage can also occur independent of caspase(Yang et al., 2004). DNA damage is another typical feature of cell apoptosis. Taken together, these results suggest that piHDL has the ability to induce oxidative stress, lipid accumulation and apoptosis in human macrophages. Glutathione oxidation is a major contributor to cell apoptosis mediated by oxidants. ROS have many actions, including oxidative modification of lipids, proteins and oxidative damage of DNA(Birben et al., 2012). Thus, ROS-induced DNA damage likely leads to apoptosis. However, the detailed mechanisms by which piHDL promotes apoptosis are not clear at present.

Macrophage apoptosis can be triggered by a variety of factors that work alone or, most likely, in combination to trigger macrophage death. (Seimon and Tabas, 2009). The accumulated cholesterol or its oxidation product-oxysterol in macrophages can result in endoplasmic reticulum stress, mitochondrial dysfunction in macrophages and ultimately apoptosis(Sano and Reed, 2013)(Yao and Tabas, 2001).The present data demonstrated that, piHDL, unlike nHDL, stimulated NADPH-oxidase-mediated ROS formation, intracellular cholesterol accumulation and macrophage apoptotic death.

HDL particles display several potent antiatherogenic functions, including the unique ability of these lipoproteins to remove cholesterol from the arterial wall, anti-oxidant, anti-inflammatory, and anti-apoptotic functions (Eren et al., 2014). However, all HDL is not functionally equivalent. HDL can lose its protective activities through a variety of mechanisms including, altered protein and lipid composition, oxidative modification of proteins and lipids(Smith, 2010b, Hima Bindu et al., 2011). In characterization , piHDL showed an enrichment of triglycerides, phospholipids, lipid peroxides, MMP-9 and diminished activity of paraoxonase-1, an anti-oxidant enzyme, compared to nHDL(S.Sini, 2013, S. Sini 2014). The observed impairments in HDL functionality might be associated with compositional changes in the HDL proteins and lipids, which posses potent pro-inflammatory and pro-oxidant properties. More studies are needed to determine the exact pathways and apoptosis regulatory molecules participating on this process.

Normal HDL particles display potent cytoprotective actions. The atheroprotective activity of HDL involves its ability to regulate cholesterol homeostasis and inhibition of apoptosis.The anti-apoptotic property of HDL includes preservation of mitochondrial integrity, reduced fragmentation of nuclear DNA, and activation of the autophagic pathway. However, piHDL induces ROS generation, lipid accumulation and apoptosis in macrophages. The data demonstrate a significant role of piHDL in macrophage apoptosis therby further promote lesion progression. These results suggest that, in human macrophages, nHDL and piHDL could be involved in the differential regulation of macrophage survival/cytotoxicity.

In conclusion, the present study demonstrates that nHDL from healthy subjects maintains cellular cholesterol homeostasis, ROS generation, mitochondrial membrane potential, DNA integrity, autophagy and cell survival. In contrast, piHDL from CAD patients disturbs cellular metabolism resulting in cholesterol accumulation, excess generation of ROS, membrane depolarization, DNA damage and eventually apoptosis, thus demonstrating proatherogenic property. The findings of this study suggest that piHDL in addition to its reported proatherogenic effects-prooxidant, pro-inflammatory effects , could also induce lipid accumulation and apoptosis in human monocyte-derived macrophages . Macrophage apoptosis is an important feature in atherogenesis, and is, in part, stimulated by dysfunctional HDL, thus providing new insights in our understanding of the atherogenic mechanisms. These findings may contribute to future development of therapeutics targeting HDL functionality.

SUMMARY AND CONCLUSION

Coronary artery disease(CAD) is an inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate lesions in the coronary arteries. CAD has a multi-factorial etiology, with many of the risk factors being influenced by lifestyle. Yet an abundance of epidemiological evidence identifies low high density lipoprotein-cholesterol (HDL-C) as an independent risk factor for CAD. This relationship is supported by the potential anti-atherogenic properties of HDL, including cholesterol efflux from arterial macrophages, i.e. reverse cholesterol transport, antioxidative, antiinflammatory, antithrombotic and antiapoptotic effects. However, many patients who experience a clinical event have normal or even high levels of HDL-C. Recent data indicate that under some inflammatory conditions, HDL can be modified, lose its cardio-protective effects and become dysfunctional HDL. The attenuated atheroprotective properties of HDL in metabolic diseases raise the possibility of an indirect putative proatherogenic effect of these particles. However, the underlying mechanisms responsible for generating dysfunctional HDL, the chemical and structural changes of HDL and the pro-atherogenic pathways exerted by the dysfunctional HDL remain largely unknown. The present study was aimed at (i) identifying the prevalence of dysfunctional HDL in healthy subjects and in patients having established CAD (ii) characterization of HDL together with underlying causative factors modulating HDL functionalities (iii) determining the effects of functional and dysfunctional HDL on human macrophage functions relevant to atherosclerosis, specifically oxidative stress, inflammatory response, foam cell formation and delineating the associated molecular mechanisms.

The functional assay of HDL revealed that HDL from majority of healthy subjects exhibited remarkable antioxidant property to inhibit LDL oxidation and is termed functional HDL(nHDL). However, HDL from CAD patients did not possess antioxidant activity and did not inhibit oxidation of LDL. On the contrary, it increased LDL oxidation, thereby demonstrating pro-inflammatory property, a characteristics of dysfunctional HDL(piHDL). When the functional status of circulating HDL was assessed among healthy volunteers using larger sample size, it was observed that HDL functionality varied widely, irrespective of the level of HDL-C. This finding indicates that all HDL are functionally not equivalent.

Characterisation of HDL indicates that piHDL has an altered lipidomic and proteomic constitution with enriched phospholipids, triglycerides, lipid peroxides and diminished paraoxonase-1 activity. Further characterization by gelatin zymography and western blot analysis demonstrated for the first time, the association of matrix metalloproteinase-9(MMP-9) activity selectively in the piHDL particle. These results suggest that dysfunctional HDL can acquire pro-inflammatory property, due to its content of altered proteome, lipids, its oxidation products and possibly, due to its association with the protease-MMP-9.

The next objective was focused on the pro-atherogenic effects of piHDL in comparison to nHDL, using human peripheral blood monocytes-macrophages, the predominant cells involved in atherogenesis. The results showed that piHDL treatment caused significant increase in ROS production, release of TNF-α and MMP-9 & MMP-2 compared to nHDL, indicating its pro-inflammatory property.

Macrophage foam cell formation in arterial wall is a fundamental, yet incompletely understood component in atherogenesis. The ability of HDL to promote cholesterol efflux from macrophages is an important anti-atherogenic mechanism. However, exposure of macrophages to piHDL, in contrast to nHDL, resulted in marked uptake of lipids from dysfunctional HDL, leading to formation of foam cell phenotype with concomitant increase in total cholesterol content. Subsequent investigations were focused on the mechanisms controlling the intracellular transport of lipids by dysfunctional HDL. Using western blotting and RT-PCR it was observed that piHDL profoundly upregulated the expression of scavenger receptor CD36 in macrophages compared to nHDL, thereby facilitating cholesterol influx capacity of macrophages. In contrast, the expression levels of other receptors- ABCG1 and SRB1, which mediates cellular cholesterol efflux, were found suppressed. The observed results provide clear evidence for the differential regulation of macrophage receptors involved in cholesterol homeostasis by the two forms of HDL, functional and dysfunctional HDL. It was then identified that CD36 did not act alone, but it was activated in macrophages along with ERK/MAPK, in response to piHDL, which in turn led to lipid accumulation as well as pro-inflammatory response via activation of NFkB. Lipid-laden foam cells are the characteristic pathological cells in atherosclerotic-plaques. These findings suggest that piHDL can impede macrophage-reverse cholesterol transport pathway, which inturn can increase the risk for atherosclerosis.

Subsequent experiments were aimed at investigating the fate of macrophage-foam cells and elucidating the mechanism that govern piHDL- induced cell toxicity.

Exposure to piHDL was found to enhance remarkable ROS production, mediated mainly by NADPH oxidase, with concomitant decrease in antioxidants-glutathione, superoxide dismutase and catalase, as indicative of excess oxidative stress in macrophages. Excess ROS formation as well as intracellular cholesterol accumulation was found to be toxic to cells as noted by significant cell death at 24 h in response to piHDL. Additional experiments revealed that macrophages exhibited DNA damage, mitochondrial depolarization and cleavage of PARP (apoptotic marker protein), indicative of an apoptotic mechanism involved in cell death induced by piHDL. Macrophage apoptosis is a prominent feature of atherosclerotic-lesions.

In conclusion, the present study demonstrated that HDL from CAD subjects, i.e. piHDL, in contrast to functional HDL from healthy subjects, was not atheroprotective, as it stimulates pro-atherogenic effects in human macrophages including oxidative stress, pro-inflammatory response, foam cell formation, cytotoxicity and apoptotic-cell death in foam cells. Macrophage receptors for dysfunctional HDL mediated proatherogenic effects are poorly defined so far.The present study reveals that CD36 is upregulated abundantly in response to piHDL that plays a major role in lipid accumulation. Furthermore, the HDL receptor-SRB-1 and ABCG-1 are suppressed in macrophages which play an essential role in promoting efflux of cholesterol from macrophage to HDL. Another remarkable finding in the present study is the previously unrecognized association of matrix metalloproteinase-9 with dysfunctional HDL. Macrophage foam cell formation and subsequent apoptosis are important features in atherosclerosis and are, in part, promoted by dysfunctional HDL, thus providing new insights in our understanding of the

atherogenic mechanisms. These findings suggest a novel molecular connection that can enhance the risk of atherosclerotic-CAD in subjects having dysfunctional HDL.

FUTURE DIRECTIONS

- Under particular circumstances, HDL particles can become dysfunctional independently of the levels of HDL-C, which possess potent .pro-atherogenic properties. The functional assay of HDL could lead to improved predictive accuracy of cardiovascular disease risk associated with circulating HDL.Considering high CAD risk in our population and knowing that conventional risk factors may not fully explain the excess CAD risk, further studies using larger sample size will be needed to validate the role of dysfunctional high density lipoprotein or any other component in dysfunctional HDL, as a CAD risk marker
- Proinflammatory potential of HDL, appears to be reversible. Therapeutic interventions addressing reversal of HDL functionality need to be established. Strategies that may improve the antioxdative/anti-inflammatory properties of HDL, includes lifestyle factors, and/ or pharmacologic approaches,eg apoA1 mimetic peptides or natural products, are to be investigated using ex-vivo model system or animal studies
- A key early event in atherosclerosis is endothelial dysfunction. HDL has diverse anti-inflammatory action in Endothelial cells.A new dimension in the anti-atherogenic function of HDL is progenitor cell–mediated endothelial repair. Functional impairment in HDL might influence endothelial progenitor cell bilogy. EPC availability in circulation and its function, which is an unknown phenomenon..Future resarch on this area may give an indication of endothelial function.

BIBLIOGRAPHY

Adar, S.D., Sheppard, L., Vedal, S., Polak, J.F., Sampson, P.D., Diez Roux, A.V., Budoff, M., Jacobs, D.R., Jr., Barr, R.G., Watson, K., Kaufman, J.D., 2013. Fine particulate air pollution and the progression of carotid intima-medial thickness: a prospective cohort study from the multi-ethnic study of atherosclerosis and air pollution. PLoS medicine 10, e1001430.

Adorni, M.P., Zimetti, F., Billheimer, J.T., Wang, N., Rader, D.J., Phillips, M.C., Rothblat, G.H., 2007. The roles of different pathways in the release of cholesterol from macrophages. Journal of lipid research 48, 2453-2462.

Alexander, E.T., Tanaka, M., Kono, M., Saito, H., Rader, D.J., Phillips, M.C., 2009. Structural and functional consequences of the Milano mutation (R173C) in human apolipoprotein A-I. Journal of lipid research 50, 1409-1419.

Amarjit Singh, N.S.,Neki., Mithila Bisht. ,Sakshi Choudhry., Ishu Singh., Himanshu Gupta., 2012. Current Advances in Understanding the Pathogenesis of Atherosclerosis and its Clinical Implications in Coronary Artery Disease. Journal International Medical Sciences Academy 25,251-253

Annema, W., Nijstad, N., Tolle, M., de Boer, J.F., Buijs, R.V., Heeringa, P., van der Giet, M., Tietge, U.J., 2010. Myeloperoxidase and serum amyloid A contribute to impaired in vivo reverse cholesterol transport during the acute phase response but not group IIA secretory phospholipase A(2). Journal of lipid research 51, 743-754.

Ansell, B.J., Navab, M., Hama, S., Kamranpour, N., Fonarow, G., Hough, G., Rahmani, S., Mottahedeh, R., Dave, R., Reddy, S.T., Fogelman, A.M., 2003. Inflammatory/antiinflammatory properties of high-density lipoprotein distinguish patients from control subjects better than high-density lipoprotein cholesterol levels and are favorably affected by simvastatin treatment. Circulation 108, 2751-2756.

Antoniades, C., Tousoulis, D., Stefanadis, C., 2007. Effects of endothelial nitric oxide synthase gene polymorphisms on oxidative stress, inflammatory status, and coronary atherosclerosis: an example of a transient phenotype. Journal of the American College of Cardiology 49, 1226; author reply 1226-1227.

Aschoff, 1933. Arteriosclerosis. Survey of the Problem. Can Med Assoc J. 29, 698.

Asleh, R., Blum, S., Kalet-Litman, S., Alshiek, J., Miller-Lotan, R., Asaf, R., Rock, W., Aviram, M., Milman, U., Shapira, C., Abassi, Z., Levy, A.P., 2008. Correction of HDL dysfunction in individuals with diabetes and the haptoglobin 2-2 genotype. Diabetes 57, 2794-2800.

Assmann, G., Gotto, A.M., Jr., 2004. HDL cholesterol and protective factors in atherosclerosis. Circulation 109, III8-14.

Kumar V, Abbas AK, Fausto N, Aster JC, editors. Pathologic basis of disease. 8th ed. Pennsylvania(US): Elseviers; 2010

Babiak, J., Rudel, L.L., 1987. Lipoproteins and atherosclerosis. Bailliere's clinical endocrinology and metabolism 1, 515-550.

Bae, Y.S., Lee, J.H., Choi, S.H., Kim, S., Almazan, F., Witztum, J.L., Miller, Y.I., 2009. Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2. Circulation research 104, 210-218, 221p following 218.

Baigent, C., Blackwell, L., Emberson, J., Holland, L.E., Reith, C., Bhala, N., Peto, R., Barnes, E.H., Keech, A., Simes, J., Collins, R., 2010. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Lancet 376, 1670-1681.

Baron, A.A., Baron, S.B., 2007. High levels of HDL cholesterol do not predict protection from cardiovascular disease in women. Preventive cardiology 10, 125-127.

Barter, P.J., Nicholls, S., Rye, K.A., Anantharamaiah, G.M., Navab, M., Fogelman, A.M., 2004. Antiinflammatory properties of HDL. Circulation research 95, 764-772.

Barter PJ, Caulfield, M., Eriksson M, Scott M. Grundy, M.S , Kastelein JJP etal, 2007. Effects of Torcetrapib in Patients at High Risk for Coronary Events. The New England journal of medicine 357.

Besler, C., Heinrich, K., Rohrer, L., Doerries, C., Riwanto, M., Shih, D.M., Chroni, A., Yonekawa, K., Stein, S., Schaefer, N., Mueller, M., Akhmedov, A., Daniil, G., Manes, C., Templin, C., Wyss, C., Maier, W., Tanner, F.C., Matter, C.M., Corti, R., Furlong, C., Lusis, A.J., von Eckardstein, A., Fogelman, A.M., Luscher, T.F., Landmesser, U., 2011. Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. The Journal of clinical investigation 121, 2693-2708.

Besler, C., Luscher, T.F., Landmesser, U., 2012. Molecular mechanisms of vascular effects of High-density lipoprotein: alterations in cardiovascular disease. EMBO molecular medicine 4, 251-268.

Beutler, E., Kelly, B.M., 1963. The effect of sodium nitrite on red cell GSH. Experientia 19, 96-97.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. The World Allergy Organization journal 5, 9-19.

Bode, W., Gomis-Ruth, F.X., Stockler, W., 1993. Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS letters 331, 134-140.

Bolann, B.J., Ulvik, R.J., 1991. Improvement of a direct spectrophotometric assay for routine determination of superoxide dismutase activity. Clinical chemistry 37, 1993-1999.

Bonetti, P.O., Lerman, L.O., Lerman, A., 2003. Endothelial dysfunction: a marker of atherosclerotic risk. Arteriosclerosis, thrombosis, and vascular biology 23, 168-175.

Borer, J.S., 2008. Heart rate: from risk marker to risk factor. European Heart Journal Supplements.

Bourcier, T., Sukhova, G., Libby, P., 1997. The nuclear factor kappa-B signaling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. The Journal of biological chemistry 272, 15817-15824.

Breton, C.V., Yin, F., Wang, X., Avol, E., Gilliland, F.D., Araujo, J.A., 2014. HDL anti-oxidant function associates with LDL level in young adults. Atherosclerosis 232, 165-170.

Brook. RD, Pope S.R., C., Brook JR, Bhatnagar A, Ana V etal 2010. Particulate Matter Air Pollution and Cardiovascular Disease .An Update to the Scientific Statement From the American Heart Association. Circulation 121.

Brouillette, C.G., Anantharamaiah, G.M., Engler, J.A., Borhani, D.W., 2001. Structural models of human apolipoprotein A-I: a critical analysis and review. Biochimica et biophysica acta 1531, 4-46.

Castelli, W.P., Garrison, R.J., Wilson, P.W., Abbott, R.D., Kalousdian, S., Kannel, W.B., 1986. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. Jama 256, 2835-2838.

Chaitanya, G.V., Steven, A.J., Babu, P.P., 2010. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. Cell communication and signaling : CCS 8, 31.

Chan, L., 1989. The apolipoprotein multigene family: structure, expression, evolution, and molecular genetics. Klinische Wochenschrift 67, 225-237.

Chen, M., Masaki, T., Sawamura, T., 2002. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. Pharmacology & therapeutics 95, 89-100.

Choi, S.H., Harkewicz, R., Lee, J.H., Boullier, A., Almazan, F., Li, A.C., Witztum, J.L., Bae, Y.S., Miller, Y.I., 2009. Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake. Circulation research 104, 1355-1363.

Cimmino, G., Ciccarelli, G., Morello, A., Ciccarelli, M., Golino, P., 2015. High Density Lipoprotein Cholesterol Increasing Therapy: The Unmet Cardiovascular Need. Translational medicine @ UniSa 12, 29-40.

Cuchel, M., Rader, D.J., 2006. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation 113, 2548-2555.

Cullen P , Rauterberg J , Lorkowski S, 2005. The Pathogenesis of Atherosclerosis. Springer-Verlag Berlin Heidelberg .170:3–70

Davidson, W.S., Silva, R.A., Chantepie, S., Lagor, W.R., Chapman, M.J., Kontush, A., 2009. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. Arteriosclerosis, thrombosis, and vascular biology 29, 870-876.

Davidson, W.S., Thompson, T.B., 2007. The structure of apolipoprotein A-I in high density lipoproteins. The Journal of biological chemistry 282, 22249-22253.

De Caterina, R., Libby, P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, M.A., Jr., Shin, W.S., Liao, J.K., 1995. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. The Journal of clinical investigation 96, 60-68.

Deanfield, J.E., Halcox, J.P., Rabelink, T.J., 2007. Endothelial function and dysfunction: testing and clinical relevance. Circulation 115, 1285-1295.

Distelmaier, K., Wiesbauer, F., Blessberger, H., Oravec, S., Schrutka, L., Binder, C., Dostal, E., Schillinger, M., Wojta, J., Lang, I.M., Maurer, G., Huber, K., Goliasch, G., 2015. Impaired antioxidant HDL function is associated with premature myocardial infarction. Eur J Clin Invest 45, 731-738.

Dodani, S., 2008. Excess coronary artery disease risk in South Asian immigrants: can dysfunctional high-density lipoprotein explain increased risk? Vascular health and risk management 4, 953-961.

Dodani, S., Dong, L., Guirgis, F.W., Reddy, S.T., 2014. Carotid intima media thickness and low high-density lipoprotein (HDL) in South Asian immigrants: could dysfunctional HDL be the missing link? Archives of medical science : AMS 10, 870-879.

Doyle, B., Caplice, N., 2007. Plaque neovascularization and antiangiogenic therapy for atherosclerosis. Journal of the American College of Cardiology 49, 2073-2080.

Drexler, H., 1997. Endothelial dysfunction: clinical implications. Progress in cardiovascular diseases 39, 287-324.

Durand, E., Scoazec, A., Lafont, A., Boddaert, J., Al Hajzen, A., Addad, F., Mirshahi, M., Desnos, M., Tedgui, A., Mallat, Z., 2004. In vivo induction of endothelial apoptosis leads to vessel thrombosis and endothelial denudation: a clue to the understanding of the mechanisms of thrombotic plaque erosion. Circulation 109, 2503-2506.

Eckardstein, A.v., 2006. Atherosclerosis: Diet and Drugs.

Eggerman, T.L., Hoeg, J.M., Meng, M.S., Tombragel, A., Bojanovski, D., Brewer, H.B., Jr., 1991. Differential tissue-specific expression of human apoA-I and apoA-II. Journal of lipid research 32, 821-828.

El-Yassimi, A., Hichami, A., Besnard, P., Khan, N.A., 2008. Linoleic acid induces calcium signaling, Src kinase phosphorylation, and neurotransmitter release in mouse CD36-positive gustatory cells. The Journal of biological chemistry 283, 12949-12959.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicologic pathology 35, 495-516.

Enas, E.A., Kuruvila, A., Khanna, P., Pitchumoni, C.S., Mohan, V., 2013. Benefits & risks of statin therapy for primary prevention of cardiovascular disease in Asian Indians - a population with the highest risk of premature coronary artery disease & diabetes. The Indian journal of medical research 138, 461-491.

Eren, E., Yilmaz, N., Aydin, O., 2012. High Density Lipoprotein and it's Dysfunction. The open biochemistry journal 6, 78-93.

Eren, E., Yilmaz, N., Aydin, O., Ellidag, H.Y., 2014. Anticipatory role of high density lipoprotein and endothelial dysfunction: an overview. The open biochemistry journal 8, 100-106.

Eriksson, E.E., Xie, X., Werr, J., Thoren, P., Lindbom, L., 2001. Importance of primary capture and L-selectin-dependent secondary capture in leukocyte accumulation in inflammation and atherosclerosis in vivo. The Journal of experimental medicine 194, 205-218.

Feng, J., Han, J., Pearce, S.F., Silverstein, R.L., Gotto, A.M., Jr., Hajjar, D.P., Nicholson, A.C., 2000. Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-gamma. Journal of lipid research 41, 688-696.

Ferretti, G., Bacchetti, T., Negre-Salvayre, A., Salvayre, R., Dousset, N., Curatola, G., 2006. Structural modifications of HDL and functional consequences. Atherosclerosis 184, 1-7.

Finn A.V, Nakano M., Narula J, Kolodgie F D, Virmani R, 2010. Concept of Vulnerable/Unstable Plaque. Arteriosclerosis, Thrombosis, and Vascular Biology. 30, 1282-1292

Fisher, E.A., Feig, J.E., Hewing, B., Hazen, S.L., Smith, J.D., 2012. High-density lipoprotein function, dysfunction, and reverse cholesterol transport. Arteriosclerosis, thrombosis, and vascular biology 32, 2813-2820.

Fox, K., Ford, I., Steg, P.G., Tendera, M., Robertson, M., Ferrari, R., investigators, B., 2008. Heart rate as a prognostic risk factor in patients with coronary artery disease and left-ventricular systolic dysfunction (BEAUTIFUL): a subgroup analysis of a randomised controlled trial. Lancet 372, 817-821

Friedewald, W.T., Levy, R.I., Fredrickson, D.S., 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical chemistry 18, 499-502.

Fridovich, I., 1978. The biology of oxygen radicals. Science 201, 875-880.

Galis, Z.S., Khatri, J.J., 2002. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circulation research 90, 251-262.

Galkina, E., Ley, K., 2009. Immune and inflammatory mechanisms of atherosclerosis . Annual review of immunology 27, 165-197.

GBD 2013 Mortality and Causes of Death, Collaborators (17 December 2014). "Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013.".*Lancet* 385 (9963): 117–71.

George, R., Harikrishnan, Sivadasanpillai, Narayani Jayakumari, Anugya Bhatt, V. Thulaseedharan, J., A. Tharakan, J., 2015. Circulating Thrombotic Risk Factors in Young Patients with Coronary Artery Disease Who Are on Statins and Anti-platelet Drugs. Ind J Clin Biochem.

Glass, C.K., Witztum, J.L., 2001. Atherosclerosis. the road ahead. Cell 104, 503-516.

Glomset, J.A., 1968. The plasma lecithins:cholesterol acyltransferase reaction. Journal of lipid research 9, 155-167.

Goldbourt, U., Yaari, S., Medalie, J.H., 1997. Isolated low HDL cholesterol as a risk factor for coronary heart disease mortality. A 21-year follow-up of 8000 men. Arteriosclerosis, thrombosis, and vascular biology 17, 107-113.

Goldstein, J.L., Ho, Y.K., Basu, S.K., Brown, M.S., 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proceedings of the National Academy of Sciences of the United States of America 76, 333-337.

Gonzalez, M.A., Selwyn, A.P., 2003. Endothelial function, inflammation, and prognosis in cardiovascular disease. The American journal of medicine 115 Suppl 8A, 99S-106S.

Gordon, D.J., Rifkind, B.M., 1989. High-density lipoprotein--the clinical implications of recent studies. The New England journal of medicine 321, 1311-1316.

Gordon, T., Castelli, W.P., Hjortland, M.C., Kannel, W.B., Dawber, T.R., 1977. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. The American journal of medicine 62, 707-714.

Gotlieb, A.I., 2007. Atherosclerosis Exposed: The Complex Pathway to a Lethal Outcome. American Society for Investigative Pathology.

Gotto, A.M., Jr., 2001. Low high-density lipoprotein cholesterol as a risk factor in coronary heart disease: a working group report. Circulation 103, 2213-2218.

Gotto, A.M., Jr., Brinton, E.A., 2004. Assessing low levels of high-density lipoprotein cholesterol as a risk factor in coronary heart disease: a working group report and update. Journal of the American College of Cardiology 43, 717-724.

Gregory D. Sloop, M.D., 2016. The Hemorheologic-Hemodynamic Theory of Atherosclerosis--Introduction(Part1).
Blood flow online (http://www.bloodflowonline.com/)

Gupta, R., Guptha, S., Sharma, K.K., Gupta, A., Deedwania, P., 2012. Regional variations in cardiovascular risk factors in India: India heart watch. World journal of cardiology 4, 112-120.

Gupta, R., Joshi, P., Mohan, V., Reddy, K.S., Yusuf, S., 2008. Epidemiology and causation of coronary heart disease and stroke in India. Heart 94, 16-26.

Gupta, S., Gudapati, R., Gaurav, K., Bhise, M., 2013. Emerging risk factors for cardiovascular diseases: Indian context. Indian journal of endocrinology and metabolism 17, 806-814.

Hansson, G.K., 2005. Inflammation, atherosclerosis, and coronary artery disease. The New England journal of medicine 352, 1685-1695.

Hegele, R.A., 2004. A glimpse at HDL-based therapy for atherosclerosis. The Clinical biochemist. Reviews / Australian Association of Clinical Biochemists 25, 3-5.

Heo, S.H., Cho, C.H., Kim, H.O., Jo, Y.H., Yoon, K.S., Lee, J.H., Park, J.C., Park, K.C., Ahn, T.B., Chung, K.C., Yoon, S.S., Chang, D.I., 2011. Plaque rupture is a determinant of vascular events in carotid artery atherosclerotic disease: involvement of matrix metalloproteinases 2 and 9. Journal of clinical neurology 7, 69-76.

Herrick, 1912. clinical features of sudden obstruction of the coronary arteries. jama 23.

Hima Bindu G., Rao, V.S., Kakkar, V.V., 2011. Friend Turns Foe: Transformation of Anti-Inflammatory HDL to Proinflammatory HDL during Acute-Phase Response. Cholesterol 2011, 274629.

Hopkins, P.N., 2013. Molecular biology of atherosclerosis. Physiological reviews 93, 1317-1542.

Huang, M.M., Bolen, J.B., Barnwell, J.W., Shattil, S.J., Brugge, J.S., 1991. Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. Proceedings of the National Academy of Sciences of the United States of America 88, 7844-7848.

Huang, Y., DiDonato, J.A., Levison, B.S., Schmitt, D., Li, L., Wu, Y., Buffa, J., Kim, T., Gerstenecker, G.S., Gu, X., Kadiyala, C.S., Wang, Z., Culley, M.K., Hazen,

J.E., Didonato, A.J., Fu, X., Berisha, S.Z., Peng, D., Nguyen, T.T., Liang, S., Chuang, C.C., Cho, L., Plow, E.F., Fox, P.L., Gogonea, V., Tang, W.H., Parks, J.S., Fisher, E.A., Smith, J.D., Hazen, S.L., 2014. An abundant dysfunctional apolipoprotein A1 in human atheroma. Nature medicine 20, 193-203.

Izzo, C., Grillo, F., Murador, E., 1981. Improved method for determination of high-density-lipoprotein cholesterol I. Isolation of high-density lipoproteins by use of polyethylene glycol 6000. Clinical chemistry 27, 371-374.

James, C., 2013. Risk Factors for Coronary Artery Diseases: A Study Among Patients With Ischemic Heart Disease in Kerala. Heart India 1, 7-11.

Jean-Charles Fruchart, P.M.C.N., MD; Erik S.G. Stroes, MD, PhD;John J.P. Kastelein, MD, PhD; Patrick Duriez, PhD, 2004. New Risk Factors for Atherosclerosis and Patient Risk Assessment. Circulation 109.

Jefferis, B.J., Whincup, P., Welsh, P., Wannamethee, G., Rumley, A., Lennon, L., Thomson, A., Lawlor, D., Carson, C., Ebrahim, S., Lowe, G., 2010. Prospective study of matrix metalloproteinase-9 and risk of myocardial infarction and stroke in older men and women. Atherosclerosis 208, 557-563.

Jeroen Bax, B.J.G., Gerhard Hindricks, Ulf Landmesser, Christian M. Matter. Frank Ruschitzka . William Wijns, 2005. Matrix metalloproteinases and cardiovascular disease. European Heart Journal Supplements, 121-122.

Józefa D¹bek, A.K.., Zbigniew G¹sior, 2010. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- B): a new potential therapeutic target in atherosclerosis? pharmacological reports 62, 778-783.

Kampus, P., Muda, P., Kals, J., Ristimae, T., Fischer, K., Teesalu, R., Zilmer, M., 2006. The relationship between inflammation and arterial stiffness in patients with essential hypertension. International journal of cardiology 112, 46-51.

Kapur, N.K., Ashen, D., Blumenthal, R.S., 2008. High density lipoprotein cholesterol: an evolving target of therapy in the management of cardiovascular disease. Vascular health and risk management 4, 39-57.

Kennedy, M.A., Barrera, G.C., Nakamura, K., Baldan, A., Tarr, P., Fishbein, M.C., Frank, J., Francone, O.L., Edwards, P.A., 2005. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell metabolism 1, 121-131.

Khera, A.V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M.F., Jafri, K., French, B.C., Phillips, J.A., Mucksavage, M.L., Wilensky, R.L., Mohler, E.R., Rothblat, G.H., Rader, D.J., 2011. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. The New England journal of medicine 364, 127-135.

Kim, S.K., Abdelmegeed, M.A., Novak, R.F., 2006. The mitogen-activated protein kinase kinase (mek) inhibitor PD98059 elevates primary cultured rat hepatocyte

glutathione levels independent of inhibiting mek. Drug metabolism and disposition: the biological fate of chemicals 34, 683-689.

Klein, T., Bischoff, R., 2011. Physiology and pathophysiology of matrix metalloproteases. Amino acids 41, 271-290.

Kockx, M.M., De Meyer, G.R., Muhring, J., Jacob, W., Bult, H., Herman, A.G., 1998. Apoptosis and related proteins in different stages of human atherosclerotic plaques. Circulation 97, 2307-2315.

Kontush, A., Chapman, M.J., 2006. Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. Pharmacological reviews 58, 342-374.

Kosmider, B., Loader, J.E., Murphy, R.C., Mason, R.J., 2010. Apoptosis induced by ozone and oxysterols in human alveolar epithelial cells. Free radical biology & medicine 48, 1513-1524.

Krishnan, M.N., Zachariah, G., Venugopal, K., Mohanan, P.P., Harikrishnan, S., Sanjay, G., Jeyaseelan, L., Thankappan, K.R., 2016. Prevalence of coronary artery disease and its risk factors in Kerala, South India: a community-based cross-sectional study. BMC cardiovascular disorders 16, 12.

Kruth, H.S., 2002. Sequestration of aggregated low-density lipoproteins by macrophages. Current opinion in lipidology 13, 483-488.

Kruth, H.S., Jones, N.L., Huang, W., Zhao, B., Ishii, I., Chang, J., Combs, C.A., Malide, D., Zhang, W.Y., 2005. Macropinocytosis is the endocytic pathway that mediates macrophage foam cell formation with native low density lipoprotein. The Journal of biological chemistry 280, 2352-2360.

Kunzli, N., Jerrett, M., Garcia-Esteban, R., Basagana, X., Beckermann, B., Gilliland, F., Medina, M., Peters, J., Hodis, H.N., Mack, W.J., 2010. Ambient air pollution and the progression of atherosclerosis in adults. PloS one 5, e9096.

Kutty, V.R., Balakrishnan, K.G., Jayasree, A.K., Thomas, J., 1993. Prevalence of coronary heart disease in the rural population of Thiruvananthapuram district, Kerala, India. International journal of cardiology 39, 59-70.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods in enzymology 186, 464-478.

Lewis, G.F., 2006. Determinants of plasma HDL concentrations and reverse cholesterol transport. Current opinion in cardiology 21, 345-352.

Lewington, S., Whitlock, G., Clarke, R., Sherliker, P., Emberson, J., Halsey, J., Qizilbash, N., Peto, R., Collins, R., 2007. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. Lancet 370, 1829-1839

Libby, P., 2002. Inflammation in atherosclerosis. Nature 420, 868-874.

Libby, P., 2008. The molecular mechanisms of the thrombotic complications of atherosclerosis. Journal of internal medicine 263, 517-527.

Libby, P., Ridker, P.M., Hansson, G.K., 2011. Progress and challenges in translating the biology of atherosclerosis. Nature 473, 317-325.

Libby, P., Ridker, P.M., Hansson, G.K., Leducq Transatlantic Network on, A., 2009. Inflammation in atherosclerosis: from pathophysiology to practice. Journal of the American College of Cardiology 54, 2129-2138.

Lindstedt, L., Saarinen, J., Kalkkinen, N., Welgus, H., Kovanen, P.T., 1999. Matrix metalloproteinases-3, -7, and -12, but not -9, reduce high density lipoprotein-induced cholesterol efflux from human macrophage foam cells by truncation of the carboxyl terminus of apolipoprotein A-I. Parallel losses of pre-beta particles and the high affinity component of efflux. The Journal of biological chemistry 274, 22627-22634.

Loftus, I.M., Naylor, A.R., Goodall, S., Crowther, M., Jones, L., Bell, P.R., Thompson, M.M., 2000. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. Stroke; a journal of cerebral circulation 31, 40-47.

Lorentz, K., Flatter, B., Augustin, E., 1979. Arylesterase in serum: elaboration and clinical application of a fixed-incubation method. Clinical chemistry 25, 1714-1720.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of biological chemistry 193, 265-275.

Lund-Katz, S., Phillips, M.C., 2010. High density lipoprotein structure-function and role in reverse cholesterol transport. Sub-cellular biochemistry 51, 183-227.

Mahdy Ali, K., Wonnerth, A., Huber, K., Wojta, J., 2012. Cardiovascular disease risk reduction by raising HDL cholesterol--current therapies and future opportunities. British journal of pharmacology 167, 1177-1194.

Maiolino G, Rossitto G, Caielli P, Bisogni V, Rossi GP, Calò LA., 2013. The Role of Oxidized Low-Density Lipoproteins in Atherosclerosis: The Myths and the Facts. Mediators of Inflammation 2013.

Manjunath, C.N., Rawal, J.R., Irani, P.M., Madhu, K., 2013. Atherogenic slipidemia. Indian journal of endocrinology and metabolism 17, 969-976.

Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. Molecular cloning: a laboratory manual 545.

Marado, A.S., 2006. Biomarkers of Cardiometabolic Risk, Inflammation and Disease(http://www.springer.com/).

Marsche, G., Levak-Frank, S., Quehenberger, O., Heller, R., Sattler, W., Malle, E., 2001. Identification of the human analog of SR-BI and LOX-1 as receptors for

hypochlorite-modified high density lipoprotein on human umbilical venous endothelial cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 15, 1095-1097.

Martyniszyn, L., Szulc-Dabrowska, L., Boratynska-Jasinska, A., Struzik, J., Winnicka, A., Niemialtowski, M., 2013. Crosstalk between autophagy and apoptosis in RAW 264.7 macrophages infected with ectromelia orthopoxvirus. Viral immunology 26, 322-335.

Mecchia, D., Lavezzi, A.M., Mauri, M., Matturri, L., 2009. Feto-placental atherosclerotic lesions in intrauterine fetal demise: role of parental cigarette smoking. The open cardiovascular medicine journal 3, 51-56.

Miller, Y.I., Chang, M.K., Binder, C.J., Shaw, P.X., Witztum, J.L., 2003. Oxidized low density lipoprotein and innate immune receptors. Current opinion in lipidology 14, 437-445.

Miller, Y.I., Choi, S.H., Fang, L., Tsimikas, S., 2010. Lipoprotein modification and macrophage uptake: role of pathologic cholesterol transport in atherogenesis. Sub-cellular biochemistry 51, 229-251.

Mineo, C., Deguchi, H., Griffin, J.H., Shaul, P.W., 2006. Endothelial and antithrombotic actions of HDL. Circulation research 98, 1352-1364.

Moestrup, S.K., Kozyraki, R., 2000. Cubilin, a high-density lipoprotein receptor. Current opinion in lipidology 11, 133-140.

Mohan, V., Sandeep, S., Deepa, R., Shah, B., Varghese, C., 2007. Epidemiology of type 2 diabetes: Indian scenario. The Indian journal of medical research 125, 217-230.

Mohandas, J., Marshall, J.J., Duggin, G.G., Horvath, J.S., Tiller, D.J., 1984. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. Biochemical pharmacology 33, 1801-1807.

Mohinder Bansal, N.K.-. 2014. Oxidative Stress Mechanisms and their Modulation. Science.

Monteiro, C.E.T.B., 2008. Acidic Environment Evoked by Chronic Stress: A Novel Mechanism to Explain Atherogenesis. . Infarct Combat Project (http://www.infarctcombat.org/)

Moron, M.S., Depierre, J.W., Mannervik, B., 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochimica et biophysica acta 582, 67-78.

Morrisett, J.D., 2000. The role of lipoprotein[a] in atherosclerosis. Current atherosclerosis reports 2, 243-250.

Musunuru, K., 2010. Atherogenic dyslipidemia: cardiovascular risk and dietary intervention. Lipids 45, 907-914.

Nakajima, T., Origuchi, N., Matsunaga, T., Kawai, S., Hokari, S., Nakamura, H., Inoue, I., Katayama, S., Nagata, A., Komoda, T., 2000. Localization of oxidized HDL in atheromatous plaques and oxidized HDL binding sites on human aortic endothelial cells. Annals of clinical biochemistry 37 (Pt 2), 179-186.

National Cholesterol Education Program Expert Panel on Detection, E., Treatment of High Blood Cholesterol in, A., 2002. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation 106, 3143-3421.

Navab, M., Ananthramaiah, G.M., Reddy, S.T., Van Lenten, B.J., Ansell, B.J., Fonarow, G.C., Vahabzadeh, K., Hama, S., Hough, G., Kamranpour, N., Berliner, J.A., Lusis, A.J., Fogelman, A.M., 2004. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. Journal of lipid research 45, 993-1007.

Navab, M., Ananthramaiah, G.M., Reddy, S.T., Van Lenten, B.J., Ansell, B.J., Hama, S., Hough, G., Bachini, E., Grijalva, V.R., Wagner, A.C., Shaposhnik, Z., Fogelman, A.M., 2005. The double jeopardy of HDL. Annals of medicine 37, 173-178.

Navab, M., Berliner, J.A., Watson, A.D., Hama, S.Y., Territo, M.C., Lusis, A.J., Shih, D.M., Van Lenten, B.J., Frank, J.S., Demer, L.L., Edwards, P.A., Fogelman, A.M., 1996. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arteriosclerosis, thrombosis, and vascular biology 16, 831-842.

Navab, M., Hama, S.Y., Hough, G.P., Subbanagounder, G., Reddy, S.T., Fogelman, A.M., 2001. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. Journal of lipid research 42, 1308-1317.

Navab, M., Reddy, S.T., Van Lenten, B.J., Fogelman, A.M., 2011. HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms. Nature reviews. Cardiology 8, 222-232.

Negre-Salvayre, A., Dousset, N., Ferretti, G., Bacchetti, T., Curatola, G., Salvayre, R., 2006. Antioxidant and cytoprotective properties of high-density lipoproteins in vascular cells. Free radical biology & medicine 41, 1031-1040.

Nicholls, S.J., Dusting, G.J., Cutri, B., Bao, S., Drummond, G.R., Rye, K.A., Barter, P.J., 2005. Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory vascular changes induced by a periarterial collar in normocholesterolemic rabbits. Circulation 111, 1543-1550.

Nicholson, A.C., 2004. Expression of CD36 in macrophages and atherosclerosis: the role of lipid regulation of PPARgamma signaling. Trends in cardiovascular medicine 14, 8-12.

Nofer, J.R., Levkau, B., Wolinska, I., Junker, R., Fobker, M., von Eckardstein, A., Seedorf, U., Assmann, G., 2001. Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. The Journal of biological chemistry 276, 34480-34485.

Ogden, R.C., Adams, D.A., 1987. Electrophoresis in agarose and acrylamide gels. Methods in enzymology 152, 61-87.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical biochemistry 95, 351-358.

Olagnier, D., Lavergne, R.A., Meunier, E., Lefevre, L., Dardenne, C., Aubouy, A., Benoit-Vical, F., Ryffel, B., Coste, A., Berry, A., Pipy, B., 2011. Nrf2, a PPARgamma alternative pathway to promote CD36 expression on inflammatory macrophages: implication for malaria. PLoS pathogens 7, e1002254.

Osterud, B., Bjorklid, E., 2003. Role of monocytes in atherogenesis. Physiological reviews 83, 1069-1112.

Oya, F., Oya, T., Toda, K., 1974. Serum lipoprotein estimation by polyacrylamide gel disc electrophoresis. Nagoya journal of medical science 37, 23-27.

Packard, C.J., Shepherd, J., 1997. Lipoprotein heterogeneity and apolipoprotein B metabolism. Arteriosclerosis, thrombosis, and vascular biology 17, 3542-3556.

Paneni, F., Cosentino, F., Marrara, F., Palano, F., Capretti, G., Gregori, M., Tocci, G., Testa, M., Volpe, M., 2012. The clinical relevance of dysfunctional HDL in patients with coronary artery disease: a 3-year follow-up study. International journal of cardiology 158, 158-160.

Parhami, F., Fang, Z.T., Fogelman, A.M., Andalibi, A., Territo, M.C., Berliner, J.A., 1993. Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. The Journal of clinical investigation 92, 471-478.

Parthasarathy, S., Raghavamenon, A., Garelnabi, M.O., Santanam, N., 2010. Oxidized low-density lipoprotein. Methods in molecular biology 610, 403-417.

Pate, M., Damarla, V., Chi, D.S., Negi, S., Krishnaswamy, G., 2010. Endothelial cell biology: role in the inflammatory response. Advances in clinical chemistry 52, 109-130.

Pekka Puska; Bo Norrving; World Health Organization (2011). Global Atlas on Cardiovascular Disease Prevention and Control (PDF). World Health Organization in collaboration with the World Heart Federation and the World Stroke Organization. pp. 3–18.

Phillips, M.C., 2013. New insights into the determination of HDL structure by apolipoproteins: Thematic review series: high density lipoprotein structure, function, and metabolism. Journal of lipid research 54, 2034-2048.

Pober, J.S., Sessa, W.C., 2007. Evolving functions of endothelial cells in inflammation. Nature reviews. Immunology 7, 803-815.

Polvani, S., Tarocchi, M., Galli, A., 2012. PPARgamma and Oxidative Stress: Con(beta) Catenating NRF2 and FOXO. PPAR research 2012, 641087.

Pope, C.A., 3rd, Burnett, R.T., Thurston, G.D., Thun, M.J., Calle, E.E., Krewski, D., Godleski, J.J., 2004. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. Circulation 109, 71-77.

Prabhakaran, D., Chaturvedi, V., Shah, P., Manhapra, A., Jeemon, P., Shah, B., Reddy, K.S., 2007. Differences in the prevalence of metabolic syndrome in urban and rural India: a problem of urbanization. Chronic illness 3, 8-19.

Quazi, F., Molday, R.S., 2013. Differential phospholipid substrates and directional transport by ATP-binding cassette proteins ABCA1, ABCA7, and ABCA4 and disease-causing mutants. The Journal of biological chemistry 288, 34414-34426.

Ramanathan, G., Yin, F., Speck, M., Tseng, C.H., Brook, J.R., Silverman, F., Urch, B., Brook, R.D., Araujo, J.A., 2015. Effects of urban fine particulate matter and ozone on HDL functionality. Particle and fibre toxicology 13, 26.

Reiss, A.B., Cronstein, B.N., 2012. Regulation of foam cells by adenosine. Arteriosclerosis, thrombosis, and vascular biology 32, 879-886.

Ricketts, T.H., 2001. The matrix matters: effective isolation in fragmented landscapes. The American naturalist 158, 87-99.

Ridker, P.M., Cushman, M., Stampfer, M.J., Tracy, R.P., Hennekens, C.H., 1997. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. The New England journal of medicine 336, 973-979.

Robinet, P., Wang, Z., Hazen, S.L., Smith, J.D., 2010. A simple and sensitive enzymatic method for cholesterol quantification in macrophages and foam cells. Journal of lipid research 51, 3364-3369.

Rodrigo, E., Lopez-Hoyos, M., Escallada, R., Fernandez-Fresnedo, G., Ruiz, J.C., Pinera, C., Cotorruelo, J.G., Zubimendi, J.A., de Francisco, A.L., Arias, M., 2000. Circulating levels of matrix metalloproteinases MMP-3 and MMP-2 in renal transplant recipients with chronic transplant nephropathy. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 15, 2041-2045.

Rosenson, R.S., Brewer, H.B., Jr., Chapman, M.J., Fazio, S., Hussain, M.M., Kontush, A., Krauss, R.M., Otvos, J.D., Remaley, A.T., Schaefer, E.J., 2011. HDL

measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events. Clinical chemistry 57, 392-410.

Rosenson, R.S., Brewer, H.B., Jr., Davidson, W.S., Fayad, Z.A., Fuster, V., Goldstein, J., Hellerstein, M., Jiang, X.C., Phillips, M.C., Rader, D.J., Remaley, A.T., Rothblat, G.H., Tall, A.R., Yvan-Charvet, L., 2012. Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation 125, 1905-1919.

Ross, R., 1986. The pathogenesis of atherosclerosis--an update. The New England journal of medicine 314, 488-500.

Ross, R., 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362, 801-809.

Rye, K.A., Bursill, C.A., Lambert, G., Tabet, F., Barter, P.J., 2009. The metabolism and anti-atherogenic properties of HDL. Journal of lipid research 50 Suppl, S195-200.

Sakakura, K., Nakano, M., Otsuka, F., Ladich, E., Kolodgie, F.D., Virmani, R., 2013. Pathophysiology of atherosclerosis plaque progression. Heart, lung & circulation 22, 399-411.

Sampson, U.K., Fazio, S., Linton, M.F., 2012. Residual cardiovascular risk despite optimal LDL cholesterol reduction with statins: the evidence, etiology, and therapeutic challenges. Current atherosclerosis reports 14, 1-10.

Sano, R., Reed, J.C., 2013. ER stress-induced cell death mechanisms. Biochimica et biophysica acta 1833, 3460-3470.

Sayols-Baixeras, S., Lluis-Ganella, C., Lucas, G., Elosua, R., 2014. Pathogenesis of coronary artery disease: focus on genetic risk factors and identification of genetic variants. The application of clinical genetics 7, 15-32.

Segrest, J.P., Jones, M.K., De Loof, H., Brouillette, C.G., Venkatachalapathi, Y.V., Anantharamaiah, G.M., 1992. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. Journal of lipid research 33, 141-166.

Seimon, T., Tabas, I., 2009. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. Journal of lipid research 50 Suppl, S382-387.

Serra-Perez, A., Verdaguer, E., Planas, A.M., Santalucia, T., 2008. Glucose promotes caspase-dependent delayed cell death after a transient episode of oxygen and glucose deprivation in SH-SY5Y cells. Journal of neurochemistry 106, 1237-1247.

Shah, A., Kanaya, A.M., 2014. Diabetes and associated complications in the South Asian population. Current cardiology reports 16, 476.

Shah, A.S., Tan, L., Long, J.L., Davidson, W.S., 2013. Proteomic diversity of high density lipoproteins: our emerging understanding of its importance in lipid transport and beyond. Journal of lipid research 54, 2575-2585.

Shah, P.K., 2011. Residual risk and high-density lipoprotein cholesterol levels: is there a relationship? Reviews in cardiovascular medicine 12, e55-59.

Silverstein, R.L., Febbraio, M., 2009. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Science signaling 2, re3.

Silverstein, R.L., Li, W., Park, Y.M., Rahaman, S.O., 2010. Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. Transactions of the American Clinical and Climatological Association 121, 206-220.

Singh, N.P., Stephens, R.E., 1997. Microgel electrophoresis: sensitivity, mechanisms, and DNA electrostretching. Mutation research 383, 167-175.

Singh S, Neki NS., Bisht M, Choudhry S,Singh I, Gupta H, 2012a. Current Advances in Understanding the Pathogenesis of Atherosclerosis and its Clinical Implications in Coronary Artery Disease. JIMSA 25, 251-253.

Sinha, A.K., 1972. Colorimetric assay of catalase. Analytical biochemistry 47, 389-394.

Sini, S., Jayakumari, N., 2013. Functionally defective high density lipoprotein is pro-oxidant : a deviation from normal atheroprotective character. International Journal of Nutrition and Food Sciences 2, 92-101.

Sini, S., Deepa, D., Harikrishnan, S., Jayakumari., N, 2014. Evidence for an exclusive association of matrix metalloproteinase-9 with dysfunctional high-density lipoprotein: A novel finding. Atherosclerosis 236, 162-168.

Sirtori, C.R., 2006. HDL and the progression of atherosclerosis: new insights. European Heart Journal Supplements 8.

Skalen, K., Gustafsson, M., Rydberg, E.K., Hulten, L.M., Wiklund, O., Innerarity, T.L., Boren, J., 2002. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 417, 750-754.

Smith, J.D., 2010a. Dysfunctional HDL as a diagnostic and therapeutic target. Arteriosclerosis, thrombosis, and vascular biology 30, 151-155.

Smith, J.D., 2010b. Myeloperoxidase, inflammation, and dysfunctional high-density lipoprotein. Journal of clinical lipidology 4, 382-388.

Soman, C.R., Kutty, V.R., Safraj, S., Vijayakumar, K., Rajamohanan, K., Ajayan, K., Group, P.S., 2011. All-cause mortality and cardiovascular mortality in Kerala state of India: results from a 5-year follow-up of 161,942 rural community dwelling adults. Asia-Pacific journal of public health / Asia-Pacific Academic Consortium for Public Health 23, 896-903.

Soran, H., Hama, S., Yadav, R., Durrington, P.N., 2012. HDL functionality. Current opinion in lipidology 23, 353-366.

Soran, H., Schofield, J.D., Durrington, P.N., 2015. Antioxidant properties of HDL. Frontiers in pharmacology 6, 222.

Sorci-Thomas, M.G., Thomas, M.J., 2012. High density lipoprotein biogenesis, cholesterol efflux, and immune cell function. Arteriosclerosis, thrombosis, and vascular biology 32, 2561-2565.

Soumyarani, V.S., Jayakumari, N., 2014. Oxidatively modified high density lipoprotein promotes inflammatory response in human monocytes-macrophages by enhanced production of ROS, TNF-alpha, MMP-9, and MMP-2. Molecular and cellular biochemistry 366, 277-285.

Soumyarani, V.S., Jayakumari, N., 2014. Oxidized HDL induces cytotoxic effects: implications for atherogenic mechanism. Journal of biochemical and molecular toxicology 28, 481-489.

Srinath Reddy, K., Shah, B., Varghese, C., Ramadoss, A., 2005. Responding to the threat of chronic diseases in India. Lancet 366, 1744-1749.

Stary, H.C., Chandler, A.B., Glagov, S., Guyton, J.R., Insull, W., Jr., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J., Wagner, W.D., Wissler, R.W., 1994. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 89, 2462-2478.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztum, J.L., 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. The New England journal of medicine 320, 915-924.

Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L., Steinberg, D., 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proceedings of the National Academy of Sciences of the United States of America 81, 3883-3887.

Sternlicht, M.D., Werb, Z., 2001. How matrix metalloproteinases regulate cell behavior. Annual review of cell and developmental biology 17, 463-516.

Stirban, A., Gawlowski, T., Roden, M., 2014. Vascular effects of advanced glycation endproducts: Clinical effects and molecular mechanisms. Molecular metabolism 3, 94-108.

Sullivan, G.W., Sarembock, I.J., Linden, J., 2000. The role of inflammation in vascular diseases. Journal of leukocyte biology 67, 591-602.

Tabas, I., 2010. Macrophage death and defective inflammation resolution in atherosclerosis. Nature reviews. Immunology 10, 36-46.

Tabas, I., Williams, K.J., Boren, J., 2007. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. Circulation 116, 1832-1844.

Takeshita, S., Tokutomi, T., Kawase, H., Nakatani, K., Tsujimoto, H., Kawamura, Y., Sekine, I., 2001. Elevated serum levels of matrix metalloproteinase-9 (MMP-9) in Kawasaki disease. Clinical and experimental immunology 125, 340-344.

Tall, A.R., 2008. Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. Journal of internal medicine 263, 256-273.

Tall, A.R., Yvan-Charvet, L., Terasaka, N., Pagler, T., Wang, N., 2008. HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. Cell metabolism 7, 365-375.

Terasaka, N., Wang, N., Yvan-Charvet, L., Tall, A.R., 2007. High-density lipoprotein protects macrophages from oxidized low-density lipoprotein-induced apoptosis by promoting efflux of 7-ketocholesterol via ABCG1. Proceedings of the National Academy of Sciences of the United States of America 104, 15093-15098.

Thankappan, K.R., Shah, B., Mathur, P., Sarma, P.S., Srinivas, G., Mini, G.K., Daivadanam, M., Soman, B., Vasan, R.S., 2010. Risk factor profile for chronic non-communicable diseases: results of a community-based study in Kerala, India. The Indian journal of medical research 131, 53-63.

Thomas, A.C., Newby, A.C., 2010. Effect of matrix metalloproteinase-9 knockout on vein graft remodelling in mice. Journal of vascular research 47, 299-308.

Thorne, R.F., Mhaidat, N.M., Ralston, K.J., Burns, G.F., 2007. CD36 is a receptor for oxidized high density lipoprotein: implications for the development of atherosclerosis. FEBS letters 581, 1227-1232.

Tomas, M., Latorre, G., Senti, M., Marrugat, J., 2004. [The antioxidant function of high density lipoproteins: a new paradigm in atherosclerosis]. Revista espanola de cardiologia 57, 557-569.

Toth, M., Sohail, A., Fridman, R., 2012. Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. Methods in molecular biology 878, 121-135.

Toth, P.P., 2004. High-density lipoprotein and cardiovascular risk. Circulation 109, 1809-1812.

Tsimikas, S., Kiechl, S., Willeit, J., Mayr, M., Miller, E.R., Kronenberg, F., Xu, Q., Bergmark, C., Weger, S., Oberhollenzer, F., Witztum, J.L., 2006. Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. Journal of the American College of Cardiology 47, 2219-2228.

Tsimikas, S., Miller, Y.I., 2011. Oxidative modification of lipoproteins: mechanisms, role in inflammation and potential clinical applications in cardiovascular disease. Current pharmaceutical design 17, 27-37.

Vacek, T.P., Rehman, S., Neamtu, D., Yu, S., Givimani, S., Tyagi, S.C., 2015. Matrix metalloproteinases in atherosclerosis: role of nitric oxide, hydrogen sulfide, homocysteine, and polymorphisms. Vascular health and risk management 11, 173-183.

Varban, M.L., Rinninger, F., Wang, N., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Gosselin, M.L., Dixon, K.L., Deeds, J.D., Acton, S.L., Tall, A.R., Huszar, D., 1998. Targeted mutation reveals a central role for SR-BI in hepatic selective uptake of high density lipoprotein cholesterol. Proceedings of the National Academy of Sciences of the United States of America 95, 4619-4624.

Vepa, S., Scribner, W.M., Parinandi, N.L., English, D., Garcia, J.G., Natarajan, V., 1999. Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. The American journal of physiology 277, L150-158.

Vergeer, M., Holleboom, A.G., Kastelein, J.J., Kuivenhoven, J.A., 2010. The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis? Journal of lipid research 51, 2058-2073.

Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D., Remaley, A.T., 2011. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nature cell biology 13, 423-433.

Vickers, K.C., Remaley, A.T., 2014. HDL and cholesterol: life after the divorce? Journal of lipid research 55, 4-12.

Virchow, 1856.) Gesammalte abhandlungen zur wissenschaftlichen medtzin. Medinger Sohn & Co., Frankfurt.

Voight, B.F., Peloso, G.M., Orho-Melander, M., Frikke-Schmidt, R., Barbalic, M., Jensen, M.K., Hindy, G., Holm, H., etal .2012. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet 380, 572-580.

Wang, X., Collins, H.L., Ranalletta, M., Fuki, I.V., Billheimer, J.T., Rothblat, G.H., Tall, A.R., Rader, D.J., 2007. Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. The Journal of clinical investigation 117, 2216-2224.

Watanabe, J., Charles-Schoeman, C., Miao, Y., Elashoff, D., Lee, Y.Y., Katselis, G., Lee, T.D., Reddy, S.T., 2012. Proteomic profiling following immunoaffinity capture of high-density lipoprotein: association of acute-phase proteins and complement factors with proinflammatory high-density lipoprotein in rheumatoid arthritis. Arthritis and rheumatism 64, 1828-1837.

Watanabe, J., Chou, K.J., Liao, J.C., Miao, Y., Meng, H.H., Ge, H., Grijalva, V., Hama, S., Kozak, Reddy, S.T., 2007. Differential association of hemoglobin with proinflammatory high density lipoproteins in atherogenic/hyperlipidemic mice. A novel biomarker of atherosclerosis. The Journal of biological chemistry 282, 23698-23707.

Westerterp, M., Murphy, A.J., Wang, M., Pagler, T.A., Vengrenyuk, Y., Kappus, M.S., Gorman, D.J., Tall, A.R., 2013. Deficiency of ATP-binding cassette transporters A1 and G1 in macrophages increases inflammation and accelerates atherosclerosis in mice. Circulation research 112, 1456-1465.

WHO 2009 : World Health Organization Web site. http://www.WHO.int.

Wiehe, K., Pierce, B., Mintseris, J., Tong, W.W., Anderson, R., Chen, R., Weng, Z., 2005. ZDOCK and RDOCK performance in CAPRI rounds 3, 4, and 5. Proteins 60, 207-213.

Wilson, A.P., 2000. Cytotoxicity and viability. Animal Cell Culture: A Practical Approach 1.

Witztum, J.L., Lichtman, A.H., 2014. The influence of innate and adaptive immune responses on atherosclerosis. Annual review of pathology 9, 73-102.

Woessner, J.F., Jr., 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 5, 2145-2154.

Wright, R.S., 2013. Recent clinical trials evaluating benefit of drug therapy for modification of HDL cholesterol. Current opinion in cardiology 28, 389-398.

Yamamoto, S., Yancey, P.G., Ikizler, T.A., Jerome, W.G., Kaseda, R., Cox, B., Bian, A., Shintani, A., Fogo, A.B., Linton, M.F., Fazio, S., Kon, V., 2012. Dysfunctional high-density lipoprotein in patients on chronic hemodialysis. Journal of the American College of Cardiology 60, 2372-2379.

Yang, Y., Zhao, S., Song, J., 2004. Caspase-dependent apoptosis and -independent poly(ADP-ribose) polymerase cleavage induced by transforming growth factor beta1. The international journal of biochemistry & cell biology 36, 223-234.

Yao, P.M., Tabas, I., 2001. Free cholesterol loading of macrophages is associated with widespread mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway. The Journal of biological chemistry 276, 42468-42476.

Yu, X.H., Fu, Y.C., Zhang, D.W., Yin, K., Tang, C.K., 2013. Foam cells in atherosclerosis. Clinica chimica acta; international journal of clinical chemistry 424, 245-252.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J., Lisheng, L., Investigators, I.S., 2004. Effect of

potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 364, 937-952.

Yvan-Charvet, L., Ranalletta, M., Wang, N., Han, S., Terasaka, N., Li, R., Welch, C., Tall, A.R., 2007. Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. The Journal of clinical investigation 117, 3900-3908.

Yvan-Charvet, L., Wang, N., Tall, A.R., 2010. Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. Arteriosclerosis, thrombosis, and vascular biology 30, 139-143.

Zago, V., Sanguinetti, S., Brites, F., Berg, G., Verona, J., Basilio, F., Wikinski, R., Schreier, L., 2004. Impaired high density lipoprotein antioxidant activity in healthy postmenopausal women. Atherosclerosis 177, 203-210.

Zernecke, A., Weber, C., 2010. Chemokines in the vascular inflammatory response of atherosclerosis. Cardiovascular research 86, 192-201.

Zhang, Y., Zanotti, I., Reilly, M.P., Glick, J.M., Rothblat, G.H., Rader, D.J., 2003. Overexpression of apolipoprotein A-I promotes reverse transport of cholesterol from macrophages to feces in vivo. Circulation 108, 661-663.

LIST OF PUBLICATIONS FROM THE THESIS

- **Sini.S,** Jayakumari.N, Functionally defective high density lipoprotein is pro-oxidant: a deviation from normal atheroprotective character, International Journal of Nutrition and Food Sciences, May 2013; 2(3): 92-101

- **Sini.S**, Deepa.D, Harikrishnan.S, Jayakumari.N. Evidence for an exclusive association of matrix metalloproteinase-9 with dysfunctional high-density lipoprotein: A novel finding, Atherosclerosis 2014. 236: p. 162-168

- **Sini.S**, Deepa.D, Harikrishnan.S, Jayakumari.N. High-density lipoprotein from subjects with coronary artery disease promotes macrophage foam cell formation : role of Scavenger receptor CD36 and ERK/MAPK signaling (communicated)

- **Sini.S**, Deepa.D, Harikrishnan.S, Jayakumari.N. Differential effects of normal and dysfunctional High density lipoprotein on mitochondrial membrane potential, DNA fragmentation and apoptosis in human macrophages. (communicated)

Sini_Thesis_New.docx

ORIGINALITY REPORT

14%

SIMILARITY INDEX

PRIMARY SOURCES

1	Sini, S., D. Deepa, S. Harikrishnan, and N. Jayakumari. "Evidence for an exclusive association of matrix metalloproteinase-9 with dysfunctional high-density lipoprotein: A novel finding", Atherosclerosis, 2014. CrossCheck	2024 words — 14%
2	www.jlr.org Internet	22 words — < 1%
3	www.hindawi.com Internet	17 words — < 1%
4	www.cpcra.org Internet	17 words — < 1%
5	archive.org Internet	15 words — < 1%
6	content.onlinejacc.org Internet	15 words — < 1%

EXCLUDE QUOTES ON
EXCLUDE BIBLIOGRAPHY ON
EXCLUDE MATCHES OFF

The 14% similarity index is entirely due to a publication which was part of this thesis work.

Dr. N. Jayakumari