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HUMORAL RESPONSE TOWARDS HIGH DENSITY LIPOPROTEIN: A NEW MECHANISM FOR ATHEROGENESIS

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Thinking is more interesting than knowing, but less interesting than looking

(Johann Wolfgang von Goethe)

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ____ i

TABLE OF CONTENTS ____ iii

LIST OF FIGURES ____ vii

LIST OF TABLES ____ xi

LIST OF ABBREVIATIONS ____ xiii

ABSTRACT ____ xix

RESUMO ____ xxiii

1. INTRODUCTION ____ 1

1.1 Atherosclerosis: a global overview ____ 3

1.1.1 Hypercholesterolemia 4

1.1.2 Diet 4

1.1.3 Impaired glucose metabolism 4

1.1.4 Hypertension 5

1.1.5 Smoking 5

1.1.6 Lack of exercise 5

1.1.7 Hyperhomocysteinemia 5

1.1.8 Infections 6

1.1.9 Genetic factors 6

1.2 Lipids and Plasma Lipoproteins in Atherogenesis ____ 8

1.2.1 Lipoprotein structure and composition 8

1.2.2 Lipoprotein metabolism 9

1.2.3 Lipoproteins and cardiovascular disease (CVD) 13

1.3 High Density Lipoprotein (HDL) ____ 15

1.3.1 Structure (heterogeneity) and composition 15

1.3.2 Atheroprotective functions of HDL 27

1.3.3 Functionally defective HDL 40

1.3.4 Epidemiology of cardiovascular risk in relation to low HDL-C 43

1.3.5 Pharmacologic modulation of HDL-C 44

1.4 Immunity in Atherogenesis ____ 57

1.4.1 Overview 57

1.4.2 General innate immune response 57

1.4.3 General adaptative immune response 58

1.4.4 The immune response in atherogenesis 60
1.4.5 Immune response in atherogenesis and HDL 69
1.5 Aims of the thesis ________ 72

2. METHODS ________ 73

2.1 Immunologic-related methods ________ 75
2.1.1 Enzyme-Linked Immunoabsorbent Assays (ELISAs) 75
2.1.2 Immunoturbidimetric immunoassay 79
2.1.3 Antibody isolation 79
2.2 Biochemistry-related methods ________ 81
2.2.1 Paraoxonase 1 (PON1) activity 81
2.2.2 Total anti-oxidant capacity (TAC) 81
2.2.3 Nitric oxide metabolites 82
2.2.4 Total HDL cholesterol, HDL2 and HDL3 82
2.3 Functional assays in vitro ________ 83
2.3.1 Inhibition of paraoxonase 1 (PON1) activity by the antibodies isolated from patient's serum 83
2.3.2 In vitro exposure of human umbilical vein endothelial cells (HUVECs) to aHDL antibodies 83
2.4 Statistical analysis ________ 84

3. HUMORAL RESPONSE TOWARDS HDL COMPONENTS AND ITS ASSOCIATION WITH MODIFICATIONS OF THE ANTI-ATHEROGENIC PROPERTIES OF HDL ________ 85

3.1 aHDL and aApoA-I antibodies in systemic lupus erythematosus (SLE) ________ 87
3.2 Anti-HDL and anti-ApoA-I antibodies in non auto-immune patients with atherosclerosis-related clinical events ________ 97
3.2.1 Coronary artery disease (CAD) and ischemic stroke (IS) 97
3.2.2 Type 2 diabetes 108

4. BIOLOGIC ACTIVITY AND PATHOGENIC POTENTIAL OF ANTIBODIES TOWARDS THE HDL COMPLEX ________ 119

4.1 Introduction ________ 121
4.2 Aim ________ 121
4.3 Patients and Methods ________ 121
4.4 Isolation of aHDL and aApoA-I antibodies from patients with SLE and in vitro inhibition of PON1 activity ________ 122
4.4.1 Results 122
4.4.2 Discussion 123
4.5 Immunoaffinity chromatography purification of aHDL antibodies from patients with IS and CAD 123
4.5.1 Results 123

4.5.2 Discussion 125
4.6 In vitro inhibition of PON1 activity by aHDL antibodies ________ 125
4.6.1 Results 125
4.6.2 Discussion 125
4.7 *In vitro* exposure of human umbilical vein endothelial cells (HUVECs) to aHDL antibodies 126
4.7.1 Results 126
4.7.2 Discussion 128

5. PHARMACOLOGIC MANIPULATION OF HDL-C AND aHDL ANTIBODIES TITRES ________ 131
5.1 Exploratory, double blind placebo controlled, randomized, single cross-over study to evaluate the potential anti-oxidant activity of Niaspan® (EXPLORE) ________ 133
5.1.1 Introduction 133
5.1.2 Aim 134
5.1.3 Patients and methods 134
5.1.4 Results 137
5.1.5 Discussion 142
5.2 Prevention of atherosclerosis with atorvastatin in patients with systemic lupus erythematosus (SLE): a pilot study ________ 144
5.2.1 Introduction 144
5.2.2 Aim 145
5.2.3 Patients and methods 145
5.2.4 Results 146
5.2.5 Discussion 148
5.3 Influence of Rosuvastatin on the oxidative modification of LDL in type 2 diabetes ________ 149
5.3.1 Introduction 149
5.3.2 Aim 150
5.3.3 Patients and methods 150
5.3.4 Results 152
5.3.5 Discussion 154

6. OVERALL DISCUSSION AND PROPOSALS FOR FUTURE DIRECTIONS ________ 157

REFERENCES ________ 163

LIST OF FIGURES

Figure 1. Schematic presentation of the major lipoproteins in normal human plasma. 9

Figure 2. Overview of lipoprotein metabolism 10

Figure 3. High density lipoproteins (HDL) heterogeneity.. 17

Figure 4. Nomenclature of the high density lipoproteins (HDL) subclasses determined by different methods. 18

Figure 5. Cholesterol ester transfer protein (CETP)-mediated lipid exchange between lipoproteins.. 26

Figure 6. Summary of the key anti-atherogenic properties attributed to high density lipoprotein (HDL).. 28

Figure 7. HDL metabolism and reverse cholesterol transport (RCT) 29

Figure 8. Major pathways involved in vasodilatory activity of HDL. 34

Figure 9. Contribution of platelets to the initiation and the progression of atherosclerosis 37

Figure 10. Intracellular signalling cascate triggered by the interaction of HDL with the platelet receptors SR-B1 and ApoER2. 38

Figure 11. HDL and glucose homeostasis.. 39

Figure 12. Proposed model for generating dysfunctional HDL. 41

Figure 13. Coronary artery disease (CAD) risk predicted by high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in the Framingham Heart Study. 43

Figure 14. Residual cardiovascular risk despite statin treatment. 45

Figure 15. Percentage reductions in serum LDL cholesterol levels according to statin and daily dose. 46

Figure 16. Effect of each statin on HDL-C levels. 46

Figure 17. Mechanism of up-regulation of ApoA-I production by statins and effect on ABCA1. 47

Figure 18. Cholesterol biosynthetic pathway 48

Figure 19. Mechanism of action of fibrates. 49

Figure 20. Mechanisms of nicotinic acid-induced changes in lipolysis. 52

Figure 21. Emerging HDL-based therapies. 53

Figure 22. Interaction between innate and adaptative immunity. 59

Figure 23. T cell-dependent B cell activation. 60

Figure 24. Activation of innate immune response in atherosclerotic lesion. 61

Figure 25. Activation of T cells in atherosclerotic lesions. 63

Figure 26. Naive T-cell differentiation into specific T helper (Th) substes involved in atherosclerosis and its complex interactions. 64

Figure 27. Role of HDL and ApoA-I in regulating immune responses. 71

Figure 28: Levels of IgG aHDL (A) and IgG aApoA-I antibodies (B) in healthy controls (CTRL) and patients with systemic lupus erythematosus (SLE).. 90

Figure 29: Correlation (Spearman's rank test) between PON1 activity (U/L) and IgG aHDL levels (A) and IgG aApoA-I levels (B) in patients with systemic lupus erythematosus (SLE). 91

Figure 30: Correlation (Spearman's rank test) between the total antioxidant capacity (TAC) expressed in Vitamin E analogue (VEA) equivalent units (µM) and IgG aHDL (A), IgG aApoA-I (B) antibodies titres and PON1 activity (C) in patients with systemic lupus erythematosus (SLE). 92

Figure 31. Relationship between the titres of IgG aHDL, aApoA-I antibodies and PON1 activity with SLICC/ACR damage index (A) and disease activity (BILAG score) (B) in patients with systemic lupus erythematosus (SLE). 93

Figure 32. Relationship between the NO_2^-+NO_3^- (A) and VCAM-1 (B, C) levels with damage (SLICC/ACR DI) and disease activity (BILAG score) in patients with systemic lupus erythematosus (SLE). 94

Figure 33. Levels of IgG aHDL (A), aApoA-I (B) and aPON1 (C) antibodies in healthy controls (CTRL) and patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS). 101

Figure 34. Relationship between the titres of IgG aHDL and IgG aApo A-I (A) and aPON1 antibodies (B) in both group of patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS). 102

Figure 35. Correlation (Spearman's rank test) between PON1 activity (U/L) and IgG aApoA-I (A) and aPON1 antibodies (B) in both groups of patients with atherosclerosis-associated clinical events coronary artery disease (CAD) and ischemic stroke (IS). 103

Figure 36. Correlation (Spearman's rank test) between NO_3^-levels and IgG aHDL (A), aApoA-I (B), aPON1 (C) antibodies titres in coronary artery disease (CAD) patients. 105

Figure 37. Levels of IgG aHDL (A), aApoA-I (B) and aPON1 (C) antibodies in healthy controls (CTRL) and in patients with type 2 diabetes. Bars show the means. Mann Whitney test. 112

Figure 38. Correlation (Spearman's rank test) between glycosylated hemoglobin (HbA1c %) and IgG HDL (A), aApoA-I (B) and aPON1 (C) antibodies in patients with type 2 diabetes. 113

Figure 39. Correlation (Spearman's rank test) between PON1 activity (U/L) and IgG HDL (A), aApoA-I (B) and aPON1 (C) antibodies in patients with type 2 diabetes. 115

Figure 40. Correlation (Spearman's rank test) between VCAM-1 (ng/mL) levels and IgG aApoA-I (A) and aPON1 (B) antibodies in patients with type 2 diabetes. 116

Figure 41. *In vitro* inhibition of paraoxonase 1 (PON1) activity by aHDL (A) and aApoA-I (B) antibodies isolated from serum patients with systemic lupus erythematosus (SLE).. 122

Figure 42. Representative profile of the HDL coupling to the HiTrap NHS activated HP column at 280nm. 123

Figure 43. Representative elution profile of a pool of serum patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS) and applied to a HDL-HiTrap NHS activated HP column at 280nm.. 124

Figure 44. Levels of IgG aHDL antibodies in the fractions collected from the HDL- HiTrap NHS activated HP column after patients serum was applied (A). Capacity of recovery of aHDL antibodies from fractions 31 and 32 in relation to the original patients samples applied to the column (B). 124

Figure 45. *In vitro* inhibition of paraoxonase 1 (PON1) activity by IgG aHDL antibodies isolated from serum of patients with atherosclerosis-associated clinical events (CAD and IS).. 126

Figure 46. Effect of aHDL antibodies isolated from patient's serum on the expression of VCAM-1 in HUVECs exposed to TNF-α. 127

Figure 47. Effect of aHDL antibodies isolated from patients serum on VEGF levels produced by HUVECs.. 127

Figure 48. Study design scheme. 135

Figure 49. Effect of treatments on lipid profile. 139

Figure 50. Paraoxonase 1 (PON1) activity acording treatment groups at baseline and at end of 12 weeks of treatment. 140

Figure 51. Serum nitric oxide metabolites (NO_2^- + NO_3^-) acording treatment groups at baseline and at the end of 12 weeks of treatment. 140

Figure 52. Levels of IgG aHDL (A) and IgG aApoA-I (B) antibodies in both groups of study at baseline and at the end of 12 weeks of treatment.. 141

Figure 53. Correlation (Spearman's rank test) between IgG aApoA-I and IgG HDL antibodies (A) and HDL2 levels (B) at the end of treatment in subject taking extended-release niacin. 141

Figure 54. Correlation (Spearman's rank test) between IgGaApoA-I antibodies and PON1 activity at baseline (A) and at the end of 12 weeks of treatment (B) in subject taking extended-release niacin. 142

LIST OF TABLES

Table 1. Composition of the major lipoprotein complexes. 9

Table 2. Proteins detected in high density lipoproteins (HDL) by mass spectrometry. 15

Table 3. Classification of high density lipoproteins (HDL) by physical properties. 18

Table 4. Clinical evidence for ApoA-I/ HDL infusion. 55

Table 5. Several ligands for the different pattern-recognition receptors (PRRs), scavenger receptors (SRs) and toll-like receptors (TLRs). 61

Table 6. Demographic characteristics and clinical and serological data of healthy controls (CTRL) and patients with systemic lupus erythematosus (SLE).. 90

Table 7: Biological variables (oxidation and endothelial dysfunction markers) measured in healthy controls (CTRL) and patients with systemic lupus erythematosus (SLE). 91

Table 8: Demographic characteristics and clinical and serological data of healthy controls (CTRL) and patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS). 100

Table 9: Antibodies titres measured in healthy controls (CTRL) and in patients with atherosclerosis-associated clinical events coronary artery disease (CAD) and ischemic stroke (IS). 100

Table 10: Biological variables (oxidation and inflammation markers) measured in healthy controls (CTRL) and in patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS). 104

Table 11. Demographic characteristics and clinical and serological data of healthy controls (CTRL) and patients with type 2 diabetes. 111

Table 12. Antibodies titres measured in healthy controls (CTRL) and in patients with type 2 diabetes. 112

Table 13. Biological variables (oxidation and inflammation markers) measured in healthy controls (CTRL) and in patients with type 2 diabetes. 114

Table 14. Baseline demographic characteristics and clinical and serological data of two groups 138

Table 15. Demographic characteristics, clinical and laboratory data of patients with systemic lupus erythematosus (SLE) randomized to placebo and atorvastatin group. 147

Table 16. Biological variables mesuared of patients with systemic lupus erythematosus (SLE) randomized to placebo and atorvastatin group. 148

Table 17. Demographic characteristics, clinical and serological data of patients with type 2 diabetes randomized to receive or not rosuvastatin. 153

Table 18. Effect of six weeks treatment with rosuvastatin in serological data of patients with type 2 diabetes 153

Table 19. Biological variables mesuared after treatment with or without rosuvastatin on patients with type 2 diabetes. 154

LIST OF ABBREVIATIONS

aa - amino acid

aApoA-I - anti-apolipoprotein A-I

aApoA-II - anti-apolipoprotein A-II

aApoC-I - anti-apolipoprotein C-I

ABCA1 - adenosine triphosphate-binding cassette transporter A1

ABCG1/G4 - adenosine triphosphate-binding cassette transporter G1/G4

AC - adenylate cyclase

aCL - anti-cardiolipin

ACAT - acyl-CoA:cholesterol acyltransferase

ACE - angiotensin-converting enzyme

ACR - American College of Rheumatology

ADMA - asymmetric dimethylarginine

AGEs - advanced glycation end products

aHDL - anti-high density lipoprotein

AI-BP - apolipoprotein A-I binding protein

ALT - alanine transaminase

AMPK - 5' adenosine monophosphate-activated protein kinase

AP - alkaline phosphatase

AP-1 - activator protein-1

APCs - antigen presenting cells

APS - antiphospholipid syndrome

Apo - apolipoprotein

aPON1 - anti-paraoxonase1

ARB - angiotensin receptor blocker

AST - aspartate aminotransferase

ATGL - adipose triacylglycerol lipase

BAFF - B cell activating factor

β2GP1 - beta 2 glicoprotein 1

BCRs - B cell antigen receptor

BIC - bicarbonate

BILAG - British Isles Lupus Assessment Group

BSA - bovine serum albumin

Bregs - regulatory B cells

CAD - coronary artery disease

cAMP - cyclic adenosine monophosphate

CCL - chemokine C-C motif ligand

CCR2 - chemokine receptor type 2

CE - cholesteryl ester

CETP - cholesteryl ester transfer protein

CHD - coronary heart disease

cGMP - cyclic guanosine monophosphate

COX - cyclooxygenase

CREBP - cAMP-response element binding protein

CRP - C-reactive protein

CTRL - healthy controls

CVD - cardiovascular disease

CX3CR1 - chemokine C-X3-C motif ligand 1

DBP - diastolic blood pressure

DCs - dendritic cells

DNA - deoxyribonucleic acid

EGFR - epidermal growth factor receptor

EL - endothelial lipase

ELISA - enzyme-linked immunoabsorbent assay

EPCs - endothelial progenitor cells

eNOS - endothelial nitric oxide synthase

ET-1 - endothelin-1

FC - free cholesterol

FFA - free fatty acid

FH - familial hypercholesterolemia

FMD - flow-mediated dilation

FPG - fasting plasma glucose

FXR - farnesoid X-activated receptor

GPx3 - glutathione peroxidases isoenzyme 3

HbA1c - glycosylated haemoglobin

HBP - high density lipoprotein binding protein

Hcy - Homocysteine

HDL - high-density lipoprotein

HDL-C - high-density lipoprotein cholesterol

12-HETE - 12-hydroxyeicosatetraenoic acid

HL - hepatic lipase

HMG-CoA reductase - 3-hydroxy-3- methylglutaryl coenzyme A reductase

HOMA - homeostatic model assessment

hs-CRP - high sensitivity - C-reactive protein

HSL - hormone-sensitive lipase

HSP - heat shock proteins

HUVECs - human umbilical vein endothelial cells

ICAM-1 - intercellular adhesion molecule 1

IDL - intermediate-density lipoprotein

IgG - immunoglobulin G

IgM - immunoglobulin M

IL - interleukin

IMT - intima-media thickness

INF-γ - interferon-gamma

iNOS - inductible nitric oxide synthase

IS - ischemic stroke

KO - knockout

LCAT - lecithin:cholesterol acyltransferase

LDL - low-density lipoprotein

LDL-C - low-density lipoprotein cholesterol

LOX-1 - lectin-like oxidized LDL receptor 1

Lp(a) - lipoprotein(a)

LPL - lipoprotein lipase

Lp-PLA2 - lipoprotein-associated phospholipase A2

LPS - lipopolysaccharide

LTB_4 - leukotriene B4

LXR - liver X receptor

MAPK - mitogen-activated protein kinase

MCP-1 - monocyte chemoattractant protein

MDA - malondialdehyde

MHC - major histocompatibility complex

MI - myocardial infarction

MPO - myeloperoxidase

mRNA - messenger ribonucleic acid

MyD88 - myeloid differentiation factor 88

NAD - nicotinamide adenine dinucleotide

NADP - nicotinamide adenine dinucleotide phosphate

NADPH - nicotinamide adenine dinucleotide reduced form

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NMD - nitroglycerin-mediated endothelium-independent dilation

NK - natural killers

NO• - nitric oxide

NO_2^- - nitrite

NO_3^- - nitrate

NOS - nitric oxide synthase

3-NT - 3-nitrotyrosine

$O_2^{\bullet-}$ - superoxide anion

$ONOO^-$ - peroxynitrite

OP - organophosphate

oxLDL - oxidized low-density lipoprotein

PAF-AH - platelet-activating factor acetyl hydrolase

PAI-1 - plasminogen activator inhibitor-1

PAMPs - pathogen associated molecular patterns

PAPS - primaty antiphospholipid syndrome

PBS - phosphate buffered saline

PDGF - platelet-derived growth factor

PGI2 - prostacyclin

pI - isoelectric point

PI3K - phosphatidylinositide 3-kinases

PKA - protein kinase A

PKC - protein kinase C

PL - phospholipids

PLTP - phospholipid transfer protein

pNPP - p-nitrophenyl phosphate

PON - paraoxonase

PPAR - peroxisome proliferator-activated receptor

PRRs - pattern-recognition receptors

PUFAs - polyunsaturated fatty acids

RA - rheumatoid arthritis

RAGEs - receptor for advanced glycation end products

RCT - reverse cholesterol transport

rHDL - reconstituted particules of HDL

RXR - retinoid X receptor

S1P - sphingosine-1-phosphate

SAA - serum amyloid A

SBP - systolic blood pressure

SD - standard deviation

SLE - systemic lupus erythematosus

SLICC/ACR DI - Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index

SMCs - smooth muscle cells

SphK - sphingosine kinase

SRs - scavenger receptors

SR-BI - scavenger receptor class B type I

SREBP - sterol regulatory element-binding protein

TAC - total antioxidant capacity

TC - total cholesterol

TCRs - T-cell receptors

TF - tissue factor

TFPI - tissue factor pathway inhibitor

TG - triglycerides

TGF-β - transforming growth factor-beta

Th - T helper

TLR - toll like receptors

TNF-α - tumour necrosis factor-alpha

tPA - tissue plasmogen activator

Tregs - regulatory T cells

tRNA - transfer ribonucleic acid

TxA2 - thromboxane A2 ()

UKPDS - UK Prospective Diabetes Study

VCAM-1 - vascular cell adhesion molecule 1

VEA - vitamin E analogue

VEGF- vascular endothelial growth factor

VLDL - very low-density lipoprotein

ABSTRACT

Atherosclerosis is the major cause of morbidity and mortality in the western world. It is also responsible, directly or indirectly, for the highest percentage of health costs in most European countries. Despite the use of new technologies for the diagnosis of vascular disease and regardless of the major advances in treatment, the atherosclerosis-related clinical burden is still raising.

The “lipid theory” of atherogenesis, which identifies dyslipidemia as the primary cause of this vascular disease has some important practical implications: it allows the definition of simple guidelines and establishes therapeutic targets which can be generally met with current pharmacologic intervention.

The association between atherosclerosis an the immune system (the immune concept) has in turn provided new ways of exploring the mechanisms involved in this condition and has opened new perspectives in the understanding of the disease. However, it raises obvious difficulties when it comes to treatment options.

Of all the players (biochemical, immunological and anatomical) involved in this matter, high-density lipoproteins (HDL) are currently recognised as one of the most important factors in atherogenesis. This is based on the recognition of HDL's multiple anti-atherogenic properties: anti-oxidant, anti-inflammatory and antithrombotic, as well as its capacity to improve endothelial function. Nowadays, it is widely recognized that the anti-atherogenic functions of HDL go beyond reverse cholesterol transport (RCT), and the importance of HDL is based not just on its ability to reduce atheroma formation but also on its ability to stabilise plaques, therefore preventing their rupture and ultimately thrombosis.

Two main set of events have been recognised as fundamental in atherogenesis: one, characterized by lipoprotein metabolism alterations, resulting in pro-inflammatory and pro-oxidative lipoproteins, which interact with the normal cellular elements of the arterial wall leading to atheroma formation; the other, the immune cellular response towards new sets of antigens which lead to the production of pro-inflammatory cytokines.

Given to HDL complexity and multiple functions this lipoprotein has became a potential target for an auto-immune response, the consequences of which may explain some of the association identified in epidemiological and clinical studies, though the interaction between the immune system and HDL has never been thoroughly addressed.

Therefore, we hypothesized that under oxidative and pro-inflammatory conditions, the increase in the antigen (HDL) would lead to a consequent increase in the production of anti-HDL (aHDL) antibodies be responsible for quantitative and/or qualitative changes of HDL. The concept that these antibodies may contribute either to the long-term evolution of atherosclerosis or to the triggering of clinical events may also explain the heterogeneity found in individual patients and in large cohorts regarding risk factors and clinical outcomes. Moreover this may be a major breakthrough in understanding why therapeutic interventions that increase HDL levels, failed to show the anticipated reduction in vascular risk.

The overall aims of this thesis were to identified and characterize the humoral response towards HDL components and to evaluate the possible mechanisms that may contribute to the modifications of the anti-atherogenic properties of HDL.

To achieve this objective we investigated: **1**) the presence of aHDL antibodies in patients with systemic lupus erythematosus (SLE) and in patients with atherosclerosis-related clinical events, such as coronary artery disease (CAD), ischemic stroke (IS) and type 2 diabetes; **2**) the association between the titres of aHDL antibodies and different clinical features of these diseases; **3**) the modifications of the anti-atherogenic properties of HDL; **4**) the biologic effect of aHDL antibodies isolated from serum of patients on the anti-oxidant and anti-inflammatory properties of HDL; **5**) the effect of different pharmacologic treatments for dyslipidemia on the prevalence and activity of aHDL antibodies.

The methodologies used in this work included immunologic-related techniques (e.g. enzyme-linked immunoabsorbent assay – ELISA, immunoturbidimetric immunoassay and immunoaffinity chromatography), biochemical techniques (enzymatic assays with quantification by spectrophotometry and luminescence methods), cell culture experiments and flow cytometry.

Our results indicate that: **1**) The titres of IgG aHDL, anti-apolipoprotein A-I (aApoA-I) and anti-paraoxonase 1 (aPON1) antibodies were higher in patients with SLE, CAD, IS and type 2 diabetes when compared with age and sex matched healthy controls. **2**) The antibodies found in these patients were associated with decreased PON1 activity, (the enzyme responsible for most of the anti-oxidant effect of HDL), reduced total anti-oxidant capacity (TAC) of serum and increased biomarkers of endothelial dysfunction (nitric oxide metabolites, adhesion molecules, nitrotyrosine). In patients with SLE the antibody titres were associated with an increase in disease-related cardiovascular damage and activity

whereas in patients with type 2 diabetes they were directly related with the fasting glucose plasma (FGP) levels and the glycosylated haemoglobin (HbA1c). **3**) The antibodies isolated from serum of our patients, directly inhibited HDL-associated PON1 activity in a dose dependent way ranging from 7 to 52%. **4**) The anti-inflammatory effect of HDL, measured by the percentage of inhibition of the cytokine-induced production of vascular adhesion molecules (VCAM-1), was reduced in more than 80% by aHDL antibodies isolated from our patients. **5**) The HDL-induced angiogenesis by increasing vascular endothelial growth factor (VEGF) levels was abrogated in 65% by the antibodies isolated from serum of patients. **6**) The current available pharmacologic agents for increasing HDL-C concentrations were associated with an increase in the titres of IgG aApoA-I antibodies. This increase was higher in the extended release niacin when compared to statins probably due to their dampening effect on oxidative stress.

In conclusion, aHDL antibodies are present in different pathologic conditions. aHDL antibodies represent a family of self-reacting immunoglobulins, of which ApoA-I and PON1 might be the most relevant targets. These antibodies are biologically active, interfering with the HDL anti-oxidant and anti-inflammatory properties and, consequently, with the atherosclerotic process. The pathogenic potential of these antibodies may lead to the identification of a new biomarker for vascular disease, whilst presenting itself as a novel target for a different treatment approach which may redefine the treatment strategies and clinical trials design for HDL interventions in the future.

RESUMO

Aterosclerose é uma das principais causas de morbilidade e mortalidade no mundo ocidental. É responsável, direta ou indiretamente, pela maior percentagem de gastos com a saúde na maioria dos países europeus.

A "teoria lipídica" da aterosclerose, que se baseia na dislipidemia como causa primária para a doença vascular tem algumas implicações práticas importantes: permite a definição de linhas de orientação e protocolos simples e ainda estabelece alvos terapêuticos que podem ser atingidos na maior parte dos casos com a atual intervenção farmacológica.

A associação da aterosclerose com o sistema imunológico (a "teoria imunológica"), forneceu por sua vez novas formas de explorar os mecanismos envolvidos e abriu novas perspetivas para um conhecimento mais completo da doença. No entanto, levanta dificuldades evidentes no que diz respeito às possibilidades terapêuticas.

De todos os intervenientes no processo aterosclerótico (bioquímicos, imunológicos e anatómicos), as lipoproteínas de elevada densidade (HDL) são atualmente reconhecidas como um dos fatores mais importantes na aterogénese. Isto é baseado no reconhecimento das múltiplas propriedades anti-aterogénicas das HDL como por exemplo: a anti-oxidante, a anti-inflamatória e a antitrombótica, bem como o seu importante papel na melhoraria da função endotelial. Atualmente, é consensual que as funções anti-aterogénicas das HDL vão além do seu papel no transporte reverso do colesterol (RCT) e a importância das HDL no processo aterosclerótico baseia-se não apenas no seu papel protetor impedindo a formação da placa de ateroma, mas também na estabilização destas, prevenindo a sua ruptura e, consequentemente o evento trombótico.

Como fundamentais no processo aterosclerótico estão reconhecidos dois principais conjuntos de eventos: um caracterizado por alterações no metabolismo das lipoproteínas que resultam em lipoproteínas pró-inflamatórias e pró-oxidantes que interagem com os componentes celulares da parede arterial e que conduzem à formação da placa de ateroma; o outro evento é a resposta imunológica desencadeada contra um novo conjunto de antigénios que por sua vez leva à produção de citoquinas pró-inflamatórias.

Dada a complexidade da HDL e das suas múltiplas funções estas lipoproteínas tornaram-se um potencial alvo para a resposta auto-imune, e cujas consequências podem explicar

algumas das associações identificados em estudos clínicos e epidemiológicos. Contudo esta interação entre o sistema imunológico e HDL nunca foi exaustivamente estudada.

Portanto, pomos a hipótese de que em condições oxidativas e pró-inflamatórias, um aumento do antigénio (HDL) conduz a um consequente acréscimo na produção de anticorpos anti-HDL (aHDL) responsáveis pela alteração quantitativa e / ou qualitativa das HDL. O conceito de que estes anticorpos podem contribuir tanto para a evolução a longo prazo do processo aterosclerótico, como para o desencadeamento de eventos clínicos pode também explicar a heterogeneidade encontrada em cada doente e nos grandes estudos clínicos, no que diz respeito aos fatores de risco e *outcomes* clínicos. Para além disso, a confirmação desta hipótese pode permitir explicar porque é que as intervenções terapêuticas atualmente em desenvolvimento para aumentar os níveis de HDL, não conseguem mostrar a tão esperada redução do risco vascular.

O objetivo geral desta tese foi identificar e caracterizar a resposta humoral contra os componentes da HDL, e avaliar possíveis mecanismos que possam contribuir para a modificação das propriedades anti-aterogénicas das HDL.

Para alcançar este objetivo investigou-se: **1**) A presença de anticorpos aHDL em doentes com lúpus eritematoso sistémico (SLE) e em doentes com manifestações clínicas de aterosclerose, como os doentes com doença arterial coronária (CAD), acidente vascular cerebral isquémico (IS) e diabetes tipo 2; **2**) Os principais alvos antigénicos dentro do complexo das HDL e a associação entre os títulos de anticorpos aHDL e diferentes características clínicas destas doenças; **3**) As modificações das funções normais associadas às HDL, em particular da função anti-oxidante e anti-inflamatória; **4**) A atividade biológica dos anticorpos aHDL isolados do soro de doentes através de um conjunto de experiências *in vitro* de inibição da atividade da paraoxonase 1 (PON1) e da expressão de moléculas de adesão em culturas de células endoteliais. Para tal foi necessário estabelecer um método de isolamento dos anticorpos. Os anticorpos aHDL isolados do soro de doentes foram utilizados de forma a identificar as potenciais alterações dos sistemas celulares utilizados; **5**) O efeito de fármacos usados no tratamento das dislipidemias, em particular o ácido nicotínico e as estatinas, na variação dos títulos de anticorpos aHDL através de ensaios clínicos randomizados, controlados com placebo e em dupla ocultação.

Os métodos utilizados neste trabalho incluíram: técnicas imunológicas (como por exemplo, *enzyme-linked immunoabsorbent assay* - ELISA, ensaio imunoturbidimetrico e

cromatografia de imuno-afinidade) técnicas bioquímicas (tais como a quantificação de atividade enzimática por espectrofotometria e por luminescência), experiências com cultura de células e citometria de fluxo.

Os nossos resultados mostram que: **1**) A presença de anticorpos aHDL, e mais especificamente anticorpos contra alguns do seus principais componentes como a apolipoproteína A-I (ApoA-I, principal apolipoproteína presente nas HDL) e a PON1 (o enzima que mais contribui para a propriedade anti-oxidante das HDL), quer em doentes com doenças auto-imunes, como o SLE, quer em doentes com manifestações clínicas de aterosclerose, como CAD, IS e diabetes tipo 2. Os doentes apresentaram títulos de anticorpos IgG aHDL, aApoA-I e aPON1 significativamente mais elevados do que controlos saudáveis com a mesma idade e sexo. **2**) A correlação positiva estatisticamente significativa entre os títulos de aHDL e aApoA-I e aPON1 sugere que estes sejam dois dos principais alvos antigénicos dentro do complexo das HDL. Os anticorpos encontrados nestes doentes estão associados com a diminuição da atividade da PON1 e a uma redução da capacidade anti-oxidante total (TAC) do soro, um aumento dos biomarcadores de disfunção endotelial (como por exemplo dos metabolitos do óxido nítrico - NO_2^- e NO_3^-, as moléculas de adesão vascular e intracelular - VCAM-1 e ICAM-1 e os níveis de 3-nitrotirosina). Nos doentes com SLE os títulos destes estão associados a um aumento do dano cardiovascular e à atividade global da doença avaliados pelas escalas SLICC/ACR DI e BILAG score, respetivamente. Enquanto que nos doentes com diabetes tipo 2 estes anticorpos estão associados com um aumento dos níveis de glicemia em jejum (FGP) e hemoglobina glicada (HbA1c). **3**) Após se ter estabelecido um método de isolamento dos anticorpos que permite isolar quantidades significativas de anticorpos do soro de doentes sem perder a sua especificidade, foi identificada a capacidade dos anticorpos isolados do soro de doentes inibirem de uma forma dependente da concentração a atividade da PON1 até um máximo de 70% no caso dos doentes com SLE e ente 7-52% no caso dos anticorpos isolados de doentes com CAD e IS. **4**) O efeito anti-inflamatório das HDL na inibição da produção de VCAM-1 induzida por citoquinas (como o TNF-α) foi revertido em mais de 80% pelos anticorpos aHDL isolados do soro de doentes. **5**) A angiogenesis induzida por HDL através do aumento do fator de crescimento do endotélio vascular (VEGF) foi anulada em 65% pelos anticorpos aHDL isolados do soro de doentes. **6**) Os atuais agentes farmacológicos disponíveis para aumentar as concentrações de HDL-C estão associados a um aumento dos títulos de anticorpos

aApoA-I, sugerindo que o aumento da quantidade de HDL-C não se traduz necessariamente num aumento das funções de HDL. Este aumento foi maior no estudo clínico com niacina de liberação prolongada em comparação com os estudos com as estatinas, provavelmente devido ao seu efeito negativo sobre o stress oxidativo.

Em conclusão os anticorpos contra diversas estruturas do complexo das HDL estão presentes em diferentes condições patológicas. Os anticorpos aHDL representam uma família de imunoglobulinas, das quais a ApoA-I e a PON1 parecem ser os alvos mais relevantes. Estes anticorpos são biologicamente ativos, interferindo com as propriedades anti-oxidante e anti-inflamatórias das HDL e, consequentemente, com o processo aterosclerótico. A identificação do elevado potencial patogénico destes anticorpos poderá permitir a identificação de um novo biomarcador de risco de doença vascular, abrindo simultaneamente as portas para novas opções terapêuticas.

1. INTRODUCTION

1.1 Atherosclerosis: a global overview

Atherosclerosis derives from the Greek words athero (meaning gruel or paste) and sclerosis (hardness). It is a general term describing any hardening of medium or large arteries with consequent loss of elasticity.

Atherosclerosis is considered to be the most common cause of cardiovascular morbidity and mortality in western societies[1,2] and by 2020 atherosclerosis is expected to be the leading cause of death worldwide.[3] Atherosclerosis begin in the teenage years and progresses silently until the age 40 when it may manifest as myocardial infarction (MI) or stroke. It was initially perceived as a degenerative disease, an inevitable consequence of ageing secondary to the accumulation of lipids in the arterial wall resulting in narrowing of the lumen.

In 1913, Anitschkow and Chalatow showed that feeding cholesterol to rabbits rapidly produces an atheromatous disease similar to that found in man,[4] giving rise to the "lipid theory" of atherosclerosis.

In 1950 Gofman et al[5,6] showed that specific fractions of cholesterol such as low-density lipoprotein (LDL) were responsible for the rapid progression of atherosclerosis in human.

In the early 80s Brown and Goldstein[7] showed that circulating LDL underwent some structural modification before it became fully pro-atherogenic. In the last three decades of the 20th century Ross and Glomset[8] proposed the "response to injury hypothesis", and described atherosclerosis as consequence of mechanical, toxins, and free radicals injury to the endothelial lining of the arterial wall.[9]

The endothelium is an important organ that modulates vasomotor tone, inflammation, thrombosis.[10-13] Under normal conditions, the endothelial lining has an anti-adhesive and antithrombotic phenotype[14] but high-levels of native or modified LDL, free radicals, microorganisms, shear stress, hypertension and insulin resistance may shift the endothelium to a pro-adhesive and pro-thrombotic phenotype.

The susceptibility to atherosclerosis depends on potentially controllable factors such as hypercholesterolemia, diet, impaired glucose metabolism, hypertension, smoking, lack of exercise hyperhomocysteinemia, infections and non-modifiable risk factors such as genetic factors, age and gender.

1.1.1 Hypercholesterolemia

The concentration of serum cholesterol is determined by genetic and environmental factors such as the type and amount of fat in the diet, obesity and physical activity. Based on animal studies, epidemiologic data and interventional studies there is a strong evidence for an association between hypercholesterolemia and the increased risk of cardiovascular diseases (CVD). Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by elevated plasma of LDL cholesterol (LDL-C) levels. Mutations in the gene encoding for LDL receptor, results in a defective LDL clearance and consequent increase risk for premature CVD.[15]

Several large randomized interventional trials with lipid lowering drugs have demonstrated that the decrease of serum total cholesterol particularly of LDL-C levels led to slowing or even reversing of the progression of clinical manifestations of atherosclerosis[16-18] Conversely, serum levels of high density lipoprotein cholesterol (HDL-C) are associated with a reduction in the risk of atherosclerosis,[19] indeed therapies that raise HDL-C reduce the morbidity and mortality associated with coronary heart disease (CHD).[20,21]

1.1.2 Diet

The industrial and technological revolutions of the last 200 years has lead to mass production of food, switch food-processing techniques that have created the so-called Western diet, characterized by high intake of red meat, fat, cereals and refined sugars.[22]

Moreover the caloric intake has increased considerably, in face of clinical experimental evidences suggesting that caloric restriction may decrease the risk of atherosclerosis.[23]

1.1.3 Impaired glucose metabolism

Although hyperglycaemia is an established CVD risk factor independent of hypercholesterolemia, clinical trials, such as the UK Prospective Diabetes Study (UKPDS), have not been able to demonstrate definitively that an intensive glucose lowering policy reduces CHD events.[24-26] Thus, a focus on reducing glycaemia alone does not appear sufficient to reduce the excess risk in diabetes, highlighting the need for a comprehensive treatment of other risk factors.

Although type 2 diabetes is primarily a metabolic disorder, it is also a vascular disease[27] and the most important cause of death among these patients is CVD.[28-30] The risk of cardiovascular events including fatal CHD in diabetic patients is higher than in non-diabetics

regardless of the previous vascular history. Furthermore, the risk of cardiovascular events in diabetic patients with no previous history of vascular morbidity is similar to that of non-diabetic patients who have such a history.[31-33]

In patients with type 2 diabetes, the risk of developing atherosclerosis at an earlier age is three- to five folds greater than in non-diabetics, after controlling for other risk factors.[34-36] Although type 2 diabetes is a state of increased plasma coagulability,[37] endothelial dysfunction is the most important factor for thrombotic complications.

1.1.4 Hypertension

Hypertension, defined as a systolic blood pressure in excess of 140 mmHg or a diastolic blood pressure above 90 mmHg has been shown to accelerate atherosclerosis and the risk of cardiac and cerebrovascular disease over time.[38] The mechanism by which hypertension causes atherosclerosis is not completely known, although loss of the nitric oxide (NO•) and prostacyclin (PGI2) precede some morphologic alterations of the arterial intima.[39,40]

1.1.5 Smoking

Smoking promotes atherosclerosis by several mechanisms amongst which a higher degree of lipid peroxidation[41,42] that induces and worsens endothelial damage. Smoking is associated with an increased risk of plaque formation and a reduction in plaque stability.[42-44]

1.1.6 Lack of exercise

Physical inactivity is a recognized risk factor for atherosclerosis and there is evidence suggesting that regular exercise may decrease blood pressure and cholesterol levels, insulin resistance and excessive weight ultimately lowering atherosclerotic risk and its mortality.[45]

1.1.7 Hyperhomocysteinemia

Homocysteine (Hcy) is an amino acid (aa) with a sulphydryl group, formed by demethylation of methionine: deficiencies or impaired activity of the enzymes that metabolize Hcy as a result of genetic mutations and/or acquired deficiencies of folate, vitamin B12 and B6 causes hyperhomocysteinemia.[46] This blood levels is associated with lipid peroxidation and a pro-inflammatory state leading to endothelial damage that contributes to the development ofatherosclerosis.[47]

1.1.8 Infections

Over the past years several viral and bacterial infections have been associated with the development of atherosclerosis and the clinical complications of unstable angina, myocardial infarction, and stroke.[48,49]

Some of these mechanisms by which viruses or bacteria increase the risk of atherosclerosis include direct infection of the cells of the arterial that in turn triggers an immune response which could initiate an autoimmune process against endothelial cells.[48,50]

1.1.9 Genetic factors

Atherosclerosis is a complex multifactorial disease and it is likely that many genes may contribute to both the susceptibility and the pathogenesis of the disease. Advances in molecular genetics have revealed that genetic polymorphisms may significantly influence susceptibility to atherosclerosis. A large number of candidate genes, genetic polymorphisms and susceptibility loci have been identified in recent years and their number is rapidly increasing. Two major experimental approaches are being used to identify and understand the role of these genes: the first applies genomic and proteomic technology to study the expression, functions, and interactions of genes in models of atherosclerosis.[51] The second approach is to study human populations for genetic variations that correlate with (and may determine) differences in rates of atherogenesis.[52] Within a population, the heritability of atherosclerosis (the fraction of disease explained by genetics) has been high in most studies, frequently exceeding 50%.[51,53]

Furthermore, genes that predispose to hypertension, type 2 diabetes, endothelial dysfunction, cellular proliferation, tissue remodelling and homeostatic defects can all be considered relevant genes for atherosclerosis.

However, there is a lack of consistent results from different studies and population groups which creates an ambiguity about the role of genetic variations in the pathogenesis of atherosclerosis. Many of the individual genetic variations have only a modest effect on the risk of atherosclerotic disorders but their effects are enhanced in synergism with other genetic and environmental factors. Moreover, the variations among population groups, such as variations in age, sex, ethnicity and sample size as well as differences in clinical end points can significantly influence the results of genetic association studies.

It has become apparent that the traditional risk factors explain only part of the pathogenesis of atherosclerosis. Albeit dyslipidemia is known to be a major cause of atherosclerosis that give raise to pro-inflammatory and pro-oxidative lipoproteins it is being increasingly recognised that immunologic mechanisms are also present and both innate and adaptive immunity are implicated in the pathogenesis of atherosclerosis, [54-56]

1.2 Lipids and Plasma Lipoproteins in Atherogenesis

The major plasma lipids are cholesterol, triglycerides (TG) and phospholipids. Cholesterol is by far the most abundant sterol in plasma, predominantly synthesized by liver and other tissues but also obtained from the diet. Cholesterol is an essential component of cell membranes, a precursor of bile acids and of steroid hormones.

TG are esters formed by a glycerol backbone to which three molecules of fatty acids are attached and are stored in adipose cells.

Phospholipids are comprised of a polar head group attached to two fatty acids. Phospholipids are the most important building blocks of cell membranes, made up of bilayers of phospholipids with fatty acids oriented toward the interior of the membrane. The type of fatty acid attached to membrane phospholipids has a significant effect on membrane fluidity. The outer membrane phospholipid is mostly phosphatidylcholine whereas the inner one is phosphatidylserine.

Cholesterol, TG and phospholipids are water insoluble and because of their hydrophobicity are carried in plasma or serum on lipoproteins.

Lipoproteins are composed of lipids and proteins (apolipoproteins) at variable ratios, densities and sizes. Their role is to facilitate the transport of water insoluble lipids in the blood stream.

1.2.1 Lipoprotein structure and composition

In general lipoproteins are composed by a hydrophobic core of neutral lipids, namely cholesterol esters (CE) and TG, surrounded by a monolayer amphipathic surface of phospholipids with the fatty acids directed toward the core of the particle. Apolipoproteins and free cholesterol are embedded within the surface of the phospholipid outer layer.

The proteins component confers unique functions to each lipoprotein class by directing particle assembly, particle interaction with cell surface receptors and may acting as cofactors for enzymes involved in the metabolism of lipoproteins.[57,58] Apolipoproteins also maintain the structure of the lipoproteins by stabilizing their micellar structure.[58]

Lipoproteins are highly heterogeneous regarding to size, structure, hydrated densities, and immunological properties.[59] On the basis of the density at which they float by ultracentrifugation, lipoproteins are divided into 5 major classes: the chylomicrons are the largest and least dense and in increasing order of density can be found the very low density

lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL and HDL (Table 1 and Figure 1).

Table 1. Composition of the major lipoprotein complexes.

Complex	Source	Density (g/mL)	%Protein	%TG	%PL	%CE	%C	%FFA
Chylomicron	Intestine	<0.95	1-2	85-88	8	3	1	0
VLDL	Liver	0.95-1.006	7-10	50-55	18-20	12-15	8-10	1
IDL	VLDL	1.006-1.019	10-12	25-30	25-27	32-35	8-10	1
LDL	VLDL	1.019-1.063	20-22	10-15	20-28	37-48	8-10	1
HDL	Intestine, liver (chylomicrons and VLDLs)	1.063-1.21	33-55	3-15	26-43	15-30	2-10	0

Abbreviations: TG: triglycerides, PL: Phospholipids, CE: Cholesteryl esters, C: Free cholesterol, FFA: Free fatty acids

Two basic types of lipoproteins have been postulated. These are micellar lipoproteins and the pseudomolecular lipoproteins.[60] The micellar type contains less than 30 % of proteins and includes chylomicrons, VLDL and LDL. These are thought to consist of a hydrophobic core of TG and CE surrounded by a hydrophilic coat of protein, phospholipids, and free cholesterol. The pseudomolecular type contains more than 30 % of protein and includes HDL.

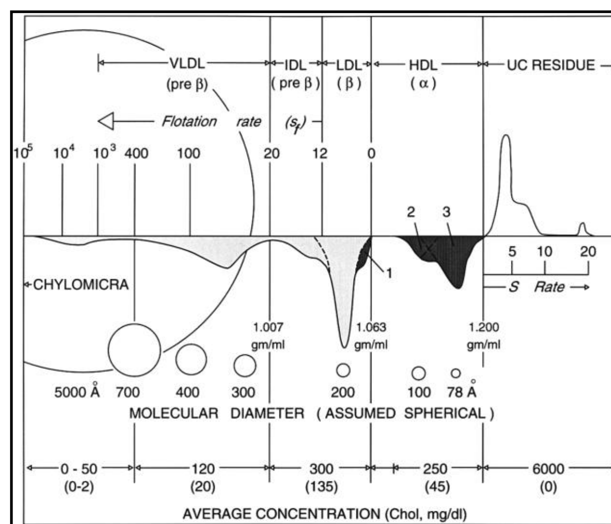


Figure 1. Schematic presentation of the major lipoproteins in normal human plasma. From Olson 1998.[61]

1.2.2 Lipoprotein metabolism

Lipoprotein metabolism involves the production, transport and removal of cholesterol and TG from the circulation, although the separation of the metabolism of these lipids is artificial as both are transported in lipoproteins. Lipid transport describes the “forward” and “reverse” transport of cholesterol. Three main pathways are responsible for the generation

and transport of lipids within the body. In this case, “forward” transport indicates the arrival of cholesterol in the blood from diet (exogenous pathway) and from liver (endogenous pathway) and the carriage back to the liver, whereas “reverse” transport (reverse cholesterol transport (RCT) pathway) is the movement of cholesterol in the opposite direction, the efflux from peripheral tissues back to the liver. They are interdependent pathways and disturbances in one will affect the function and products of the other.

Chylomicrons are the largest lipoproteins and are synthesized, assembled and secreted in the intestinal epithelial cells to transport dietary TG and cholesterol from the site of absorption to the various cells of the body (Figure 2).[62] Therefore, chylomicrons are the molecules formed to mobilize dietary lipids (exogenous pathway). The main structural apolipoprotein of chylomicrons is ApoB-48 while others may be found in small amounts.

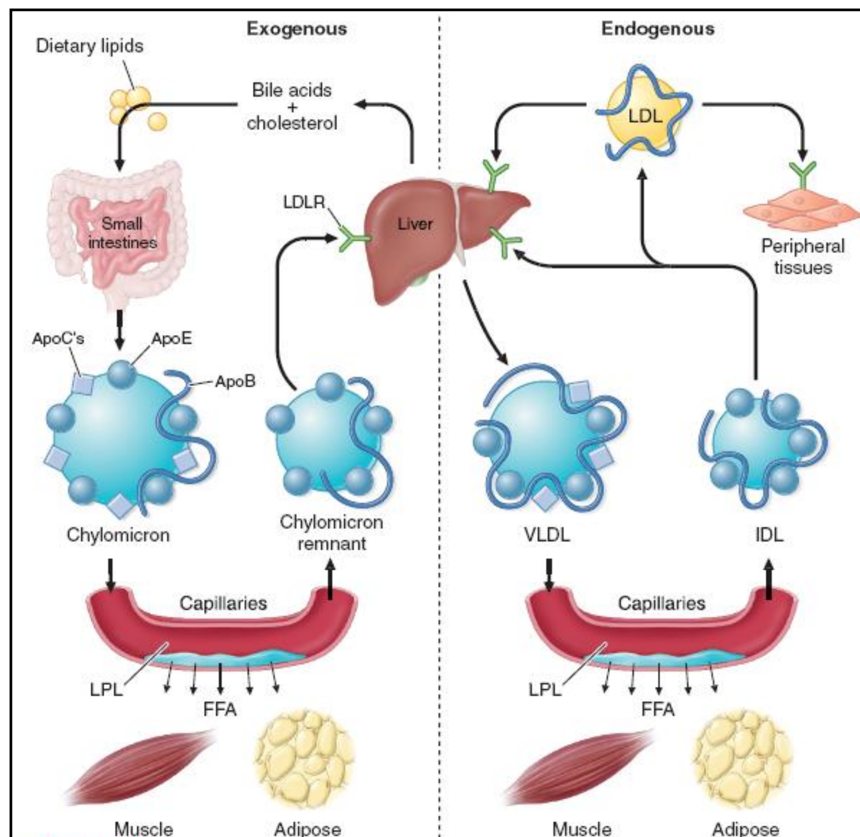


Figure 2. Overview of lipoprotein metabolism: (1) transport of diet lipids (exogenous pathway). Diet lipids are secreted from intestinal cells on chylomicrons, a process that requires apolipoprotein B (ApoB). The triglyceride (TG) within the chylomicrons is hydrolyzed by lipoprotein lipase (LPL) with apolipoprotein C-II (ApoC-II) as cofactor, producing a chylomicron remnant which is taken up by the low-density lipoprotein-like receptor protein (LRP) in the liver; (2) endogenous pathway. Very low-density lipoprotein (VLDL) are TG rich particles secreted by the liver. After suffers hydrolyses by LPL VLDL are converted in intermediate-density lipoprotein (IDL). The liver, via the interaction of ApoE with the LDL receptor, takes up some of the IDL particles; others are hydrolyzed by hepatic lipase (HL) to produce CE-rich low-density lipoprotein (LDL). LDL can be cleared by the liver via LDL receptor or delivers CE peripheral tissues such as macrophages. From Rader and Hobbs, 2008.[63]

The catabolism of chylomicrons occurs at the endothelial surfaces of capillaries in adipose tissue and muscle: the TG in the core of the chylomicrons is hydrolyzed into free fatty acids and glycerol by the action of lipoprotein lipase (LPL).[64] This free fatty acids may be then absorbed by the tissues, used as an energy source by various cells or taken up by adipocytes and stored as TG. After the action of LPL, chylomicrons shrink in size becoming chylomicron remnants with lipid cores, having a relatively high concentration of CE.

Chylomicron remnants are taken up by liver cells via receptor-mediated endocytosis by a process equivalent to the mechanism of uptake of LDL and are further metabolized by hepatic lipases (HL) releasing the cholesterol to the endoplasmic reticulum where it becomes part of the cellular cholesterol pool.[65,66] This cholesterol can be used for bile acid synthesis or resterified by acyl coenzyme A : cholesterol transferase (ACAT) and packaged along with TG within VLDL particles.

The endogenous pathway of lipoprotein metabolism refers to hepatic secretion and metabolism of VLD, IDL and LDL (Figure 2).

VLDL are TG rich particles with small amounts of CE, phospholipids, ApoB-100 and others apolipoproteins synthesized by the liver.[67] ApoB-100 also synthesized by the liver is essential for the assembly of VLDL particles and their secretion into the circulation[68,69] Like chylomicrons, VLDL acquires in the bloodstream ApoCs and ApoE from circulating HDLs. Within the plasma compartment, the TG of VLDL are hydrolyzed by LPL to free fatty acids, generating a series of smaller, cholesterol-enriched lipoproteins. The free fatty acids may be delivered to cardiac or skeletal muscle cells for β-oxidation or to adipose tissue for TG resynthesis and storage. The circulating VLDL particles become progressively smaller as their core is removed by lipolysis whereas surface materials, including phospholipids, free cholesterol, ApoC’s and some ApoE are transferred to HDL. The smaller VLDL remnant are released from the endothelial cells surface and become IDL.

IDL are essentially composed by CE and a small percentage of TG, having ApoB-100 and ApoE as their main apolipoproteins. IDL may become further enriched by CE derived from HDL by mediation of the cholesterol ester transfer protein (CETP). IDL have two metabolic fates following interaction with HL: (1) to be uptake by hepatocytes after binding to the LDL receptor in a both ApoB-100 and ApoE mediated process, or (2) are subject to further lipase activity continuing to lose TGs and are released into the circulation as LDL.[70] In humans this is the predominant pathway and ApoE is lost to HDL during both processes.

LDL are cholesterol-enriched lipoproteins, essentially CE, phospholipids, small amounts of TG, and ApoB-100 (the only protein component of LDL) make up the remainder of the particle. In comparison with the originally secreted VLDL, which range from 35 to 80 nm, LDL particles are much smaller having an average diameter of 22 nm, which allows them to cross the vascular endothelium and enter the tissue fluid delivering the cholesterol to the tissues. LDL delivers CE to peripheral (extrahepatic) cells (about 1/3 of LDL that is produced daily) or to hepatocytes within the liver (about 2/3 of LDL that is produced daily).

LDL particles are transferred from circulation to the liver and peripheral tissues via two pathways: 1) LDL receptor pathway which is regulated according to the cholesterol requirement of each individual cell; 2) non-receptor mediated pathways that depends almost entirely on the extracellular concentrations of LDL.

The LDL receptor is a single-chain transmembrane glycoprotein that is mainly expressed by hepatocytes (75 %) but is also present on adrenal and adipose tissue. Although capable of binding ApoE-containing lipoproteins, LDL receptor usually binds to the ApoB-100 - containing lipoproteins, in particular LDL. After binding the LDL receptor-ligand complex is internalised within the cell by endocytosis, where it undergoes lysosomal degradation of LDL particles. ApoB is hydrolysed to its constituent aa and the CE is hydrolysed to free cholesterol by acid lipase. Free cholesterol is released to the cytoplasm where is re-esterifed by ACAT and stored as CE, reused for lipoprotein synthesis or converted into bile acid and vitamin D.[20] Following that the receptor is recycled back to the cell surface and is again able to bind lipoproteins. Expression levels of LDL receptors are regulated by a sensitive feedback control through the intracellular cholesterol content of hepatocytes and the need for cholesterol. Transcription levels of LDL receptor gene is controlled by promoters with sterol regulatory elements, stimulation of this mechanism causes the release of a sterol regulatory element binding protein (SREBP) which binds to DNA and switches on the transcription.[71]

Excess of cellular cholesterol, for example after high intake of dietary saturated fat and cholesterol, down-regulates LDL receptor, which lowers the uptake of LDL and consequently results in elevated plasma LDL-C levels and an accumulation of cholesterol in peripheral cells. Cholesterol excess also induces suppression of microssomal 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme of cholesterol biosynthesis.[72]

Via the non-receptor-mediated pathway, LDL binds to cell membranes at sites other than those at which LDL receptors are located and some of it passes through the membrane by pinocytosis. HDL is able to compete with LDL for this type of cell-membrane association.

In addition, LDL may also be removed from the circulation by a number of receptors other than the classical LDL receptor, the scavenger receptors (CD36 and SR-A) which are responsible for the clearance of only relatively minor amounts of LDL. Since they bind modified LDL and are mainly present on the macrophages scavenger receptor have gained considerable interest because they may have a central role in atherogenesis.

Further to the exogenous and endogenous pathways above explained lipid transport also has an important third pathway, the RCT. HDL particles have the capacity to mediate cellular cholesterol efflux by acting as primary acceptors, thereby facilitating RCT, a process in which cholesterol is transferred from peripheral tissues, including from macrophages on the arterial wall, to the liver for the excretion into the bile. [73,74]

As HDL particles are the main interest of this dissertation these will be described below in more detail, including the RCT pathway since it is considered one of the most important mechanisms by which HDL protects against atherosclerosis.

1.2.3 Lipoproteins and cardiovascular disease (CVD)

The first significant study that associated lipid abnormalities with CVD risk was the Framingham Heart Study, carried out by the National Heart Institute in the small town of Framingham, Massachusetts. It begun in 1948 and it is still ongoing to this day.[75,76] The original cohort of the Framingham Heart Study consisted of 5209 subjects of a random sample of 2/3 of the adult population of Framingham with 30 to 62 years of age. Measurements were made of most of the potentially relevant risk factors for CVD known at the time. These included data on cigarette smoking, blood pressure, total serum cholesterol, LDL-C, HDL-C and presence of type 2 diabetes. Since then, the subjects have continued to return to the study every two years for a detailed medical history, physical examination, and laboratory tests with the last examination being set between May of 2008 and February of 2010.

The study provided first solid evidence that individuals with high blood cholesterol levels were more likely to have CHD. It also showed that this was true for a number of other risk

factors, such as high blood pressure, smoking, obesity, diabetes and physical inactivity, and that any combination of these risk factors were at least additive.[77]

The results of the study drived the lipid management guidelines that are still focused on decreasing LDL-C levels as the primary target for reducing CHD risk. However, despite the generalised use of statins, aggressive LDL lowering has only led to 30 % reduction of clinical events.[78] Therefore, to further reduce atherosclerotic events, increasing attention is now focused on HDL as potential target for atherosclerosis management. According to NCEP/ATP III the HDL-C goal is ≥60 mg/dL.

1.3 High Density Lipoprotein (HDL)

1.3.1 Structure (heterogeneity) and composition

Structurally, HDL represents the smallest (Stoke's diameter of 7–12 nm) and the densest (ranging from 1.063 to 1.21 /mL) plasma lipoprotein (Table 1), which is produced by the liver and small intestine.[79] It is a heterogeneous fraction, comprising a wide range of circulating particles that differ in shape, size, density, composition and surface charge/electrophoretic mobility.[80]

The basic structure of HDL involves a lipid core of CE and TG surrounded by a surface containing a phospholipid bilayer, free cholesterol and a number of apolipoproteins. HDL proteins constitute more than half of its mass, unlike the others lipoproteins which are mainly composed by cholesterol and /or TG.

Table 2. Proteins detected in high density lipoproteins (HDL) by mass spectrometry.

PROTEINs	
Albumin	Complement C3
Alfa-1-acid glycoprotein 2	Complement C4
Alfa-1-antitrypsin	Complement C4b
Alfa-1B-glycoprotein	Complement C9
Alpha-2-antiplasmin	Fibrinogen
Alpha-2-HS-glycoprotein	Haptoglobin-related protein
Alpha-2-macroglobulin	Haptoglobin
Alpha-amylase (salivary)	Inter-alpha-trypsin inhibitor chain H4
Angiotensinogen	Kininogen-1
Apo(a)	Lipopolysaccharide-binding protein
ApoA-I	LCAT
ApoA-II	PAF-AH (LpPLA2)
ApoA-IV	Platelet basic protein
ApoB	PLTP
ApoC-I	PON1
ApoC-II	PON3
ApoD	Prenylcysteine oxidise
ApoE	Prothrombin
ApoF	Retinol-binding protein
ApoH	SAA 1/2
ApoJ	SAA 4
ApoL-I	Serpin F1
ApoM	Transferrin
Bikunin	Transthyreitin
C4b binding protein	Vitamin D-binding globulin
CETP	Vitronectin

HDL proteins have traditionally been divided into four major subgroups: apolipoproteins, enzymes, lipid transfer proteins and minor proteins. However, the recent development of proteomic technologies have enhanced the sensitivity of protein detection, revealing that the protein burden of HDL is much more diverse than previously realized. [81-85]

Studies have allowed the identification of more than 50 proteins in human HDL (Table 2). In addition to the apolipoproteins and enzymes, numerous proteins involved in acute-phase response were found in HDL as well as proteins involved in complement regulation and protease inhibition,[86,87] raising the possibility that HDL may play a previously unsuspected role in host defence mechanisms and inflammation. It is important to keep in mind, however, that the content of all these proteins in HDL is much lower than that of major HDL apolipoproteins, i. e., ApoA-I and ApoA-II (70% and 20%, respectively).

1.3.1.1 HDL subpopulations

On the basis of shape most of the HDL are spherical particles, although there is also a minor subpopulation of discoidal HDL (Figure 3 a). Discoid HDL are small (≤ 8 nm) particles and represent a nascent form of HDL, being lipid-poor and presenting two or three ApoA-I molecules arranged around a phospholipid bilayer. These relatively unstable and short-lived particles will be converted into mature spherical HDL. Spherical HDL particles are larger (> 8 nm) and contain a hydrophobic core of esterified cholesterol and TG surrounded by an outer part composed by two or more ApoA-I molecules, with or without ApoA-II, phospholipids and free cholesterol.[80]

On the basis of isopycnic ultracentrifugation density HDL may be classified into three subfractions: HDL1 (mean density (d) 1.05 g/mL), HDL2 ($1.063 < d < 1.125$ g/mL) and HDL3 ($1.125 < d < 1.21$ g/mL). HDL2 and HDL3 subfractions are spherical and mature particles. The composition of the HDL3 particle is a monolayer of phospholipids, mainly phosphatidylcholine, and a small amount of free cholesterol, ApoA-I and ApoA-II and a core containing esterified cholesterol. HDL3 collects cholesterol to form the larger HDL2 particles without ApoA-II, which can exchange both cholesterol and TG with LDL and VLDL particles.

On the basis of immunoaffinity chromatography HDL may also be separated into two major subpopulations according to their apolipoprotein composition using (Figure 3 b). One subpopulation comprises HDL containing ApoA-I but no ApoA-II (LpA-I), while the other comprises particles containing both ApoA-I and ApoA-II (LpA-I/A-II).[88] ApoA-I is divided equally between LpA-I and LpA-I/A-II in most subjects, while virtually all of the ApoA-II resides in LpA-I/A-II. A small proportion of the ApoA-I exists in a lipid-poor form. LpA-I/A-II tend to be smaller and more dense than LpA-I, and prevail in the HDL3 subfraction. LpA-I are mostly found in the HDL2 subfraction.

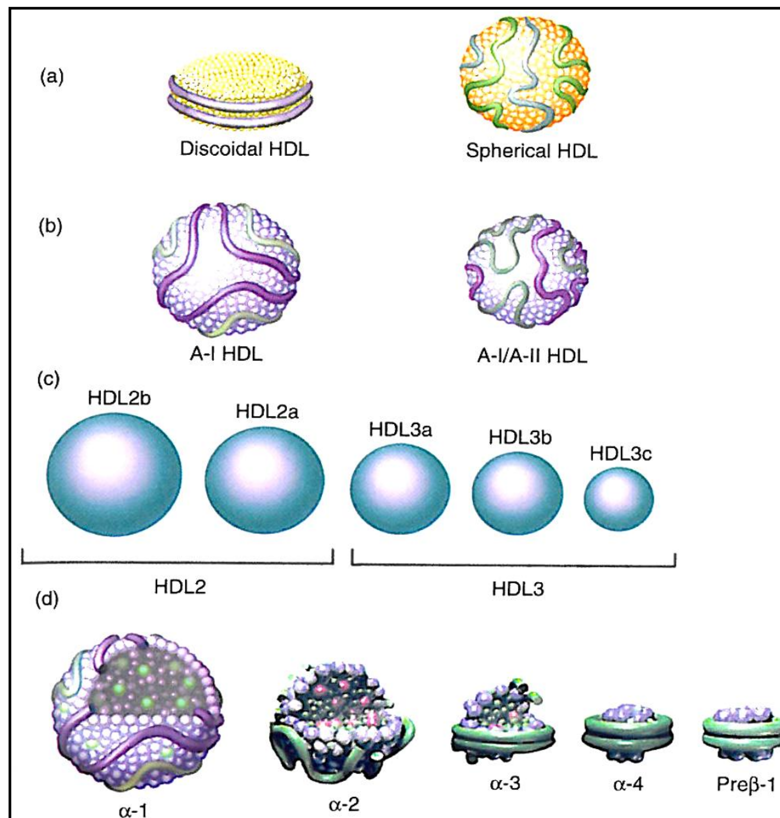


Figure 3. High density lipoproteins (HDL) heterogeneity. The HDL in human plasma consist of several subpopulations of particles which differ in shape (a), apolipoprotein composition (b), density and size (c) and surface charge in electrophoretic mobility (d). Modified from Rye KA et al 2009[89] and Tabet F & Rye KA 2009.[90]

On the basis of electrophoresis HDL can exhibit alpha (α), pre-beta (β) or gamma (γ) mobility (Figure 3 d). The α –electrophoretic mobility (faster) particles are spherical lipoproteins that include both the HDL2 and HDL3 subfractions as well as the LpA-I and LpA-I/A-II subpopulations and account for the major proportion of HDL in human plasma. Pre-β migrating (slower) HDL are either lipid-poor ApoA-I particles, with a single molecule of ApoA-I as a free molecule or in association with a few molecules of sphingomyelin and phosphatidylcholine, or nascent discoidal particles consisting of one or two molecules of ApoA-I complexed with phospholipids and possibly a small amount of unesterified cholesterol. In addition, γ migrating HDL are lipid-poor particles containing ApoE or ApoA-IV.[91,92]

The nomenclature of the HDL subpopulations varies depending on the separation technique used (Figure 4) and have generated some confusion and inconsistency when comparing different studies.

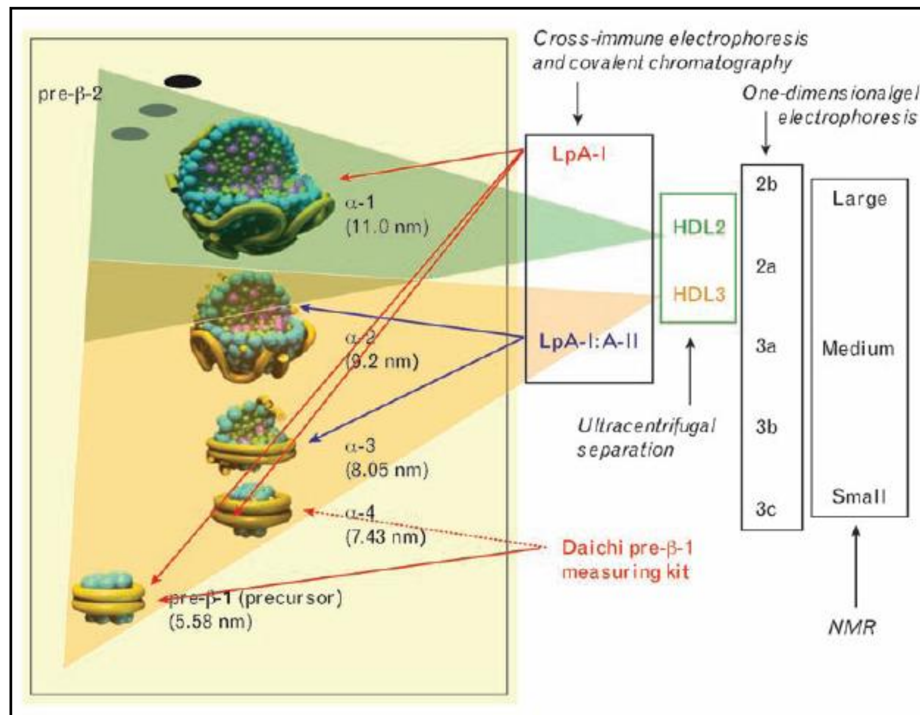


Figure 4. Nomenclature of the high density lipoproteins (HDL) subclasses determined by different methods. From Asztlos et al, 2011.[93]

Due to the increasing need to understand, validate, and quantify the diverse roles of HDL particles in the atherosclerotic process several investigators initiated an effort to uniform the nomenclature for HDL subpopulations. Recently, they proposed the development of a new classification system that defines five HDL subclasses on the basis of physical and chemical properties that includes very large HDL particles (VL-HDL), large HDL particles (L-HDL), medium HDL particles (M-HDL), small HDL particles (S-HDL), and very small HDL particles (VS-HDL). The very small HDL subclass includes pre-β-1, discoidal, or nascent HDL (Table 3).[94]

Table 3. Classification of high density lipoproteins (HDL) by physical properties. From Rosenson et al. 2011.[94]

Proposed term	Very large HDL (HDL-VL)	Large HDL (HDL-L)	Medium HDL (HDL-M)	Small HDL (HDL-S)	Very small HDL (HDL-VS)
Density range, g/mL	1.063–1.087	1.088–1.110	1.110–1.129	1.129–1.154	1.154–1.21
Size range, nm	12.9–9.7	9.7–8.8	8.8–8.2	8.2–7.8	7.8–7.2
Density gradient ultracentrifugation	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Density range, g/mL	1.063–1.087	1.088–1.110	1.110–1.129	1.129–1.154	1.154–1.170
Gradient gel electrophoresis	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Size range, nm	12.9–9.7	9.7–8.8	8.8–8.2	8.2–7.8	7.8–7.2
2-D gel electrophoresis	α-1	α-2	α-3	α-4	Pre-β-1 HDL
Size range, nm	11.2–10.8	9.4–9.0	8.5–7.5	7.5–7.0	6.0–5.0
NMR	Large HDL-P[b]	Medium HDL-P		Small HDL-P	
Size range, nm	12.9–9.7	9.7–8.8	8.8–8.2	8.2–7.8	7.8–7.2
Ion mobility	HDL2b	HDL2a and HDL3			
Size range, nm	14.5–10.5	10.5–7.65			

1.3.1.2 Major components

1.3.1.2.1 Apolipoprotein A-I (ApoA-I)

Human ApoA-I circulates in plasma primarily as a component of HDL (70 % of total HDL protein). It is also found in chylomicrons and VLDL.[95] ApoA-I has two major sites of synthesis: the intestine and the liver. The intestinal derived ApoA-I enters the circulation associated with chylomicrons but is rapidly transferred to HDL particles during lipase hydrolysis of chylomicrons. Hepatic ApoA-I enters the circulation associated with nascent HDL particles and is the major contributor to the plasma ApoA-I pool.

ApoA-I is a 28 kDa single polypeptide synthesized as a prepropeptide (267 aa residues) and is cleaved to release 24 aa residues. Mature, circulating ApoA-I consisting of 243 aa residues is encoded by exon 3 (residues 1–43) and exon 4 (residues 44–243) of a gene located on the long arm of chromosome 11. Analysis of its aa sequence reveals that with the exception of the 44 aa that form the (N)-terminal region, the protein is organized into eight α-helical amphipathic domains of 22 aa with two repeats of 11 aa that are frequently separated by proline residues. These α-helices contain the lipid-binding carboxyl (C)-terminal domain that confers ApoA-I the capacity of avidly binding to lipids and also to move between lipoproteins.[95-97] ApoA-I does not undergo post-translational modifications such as glycosylation, phosphorylation[98] and has no disulfide linkages.

ApoA-I is polymorphic in plasma and is composed of a series of isoforms of similar molecular weights but different isoelectric points (pI). The protein is made up of one major isoform ApoA-I_1 (pI 5.6) and two minor isoforms ApoA-I_2 and ApoA-I_3 (pI 5.53 and 5.46).[99,100]

The concentration of ApoA-I in plasma is about 100-150 mg/dL[58] and has a plasma half-life of about 4 days.[101]

ApoA-I exists in plasma in three general forms: lipid poor ApoA-I, nascent discoidal HDL particles and mature spherical HDL forms. [95,102,103]

In 1999 Segrest et al[104] proposed the "*double-belt model*" for discoidal HDL that consists of two ring-shaped ApoA-I molecules wrapped around a leaflet of a disk-like patch of lipid bilayer in an anti-parallel orientation. Despite some debate on details of certain regions of ApoA-I in the discs, the majority of the most recent theoretical and experimental data supports the general features of the "double belt model" [105-107]

In 1997, Borhani et al[108] hypothesized a "*Faberge Egg model*" for spherical HDL, suggesting that the intermolecular contacts found in the crystal structure (and in the

"*double-belt model*" in discs) are also present in HDL spheres. However, one model recently presented, using cross-linking chemistry and mass spectrometry , indicates that the "*double-belt model*" is a common organizational motif for ApoA-I in both discs and spheres.[109] These authors also showed that particles with a diameter superior to 93 Å (which is comparable with human HDL3) present one extra molecule of ApoA-I, but the cross-linking patterns in reconstituted spheres were highly similar to those in the discs, regardless of whether they contained three molecules of ApoA-I in each particle or only two. The most promising model seems the "*trefoil model*" where the phospholipids in the sphere surface are broken into three equal slices with angles of 120°.[109]

The understanding of the ApoA-I spatial arrangement in both discs and spheres is critical because it offers insights into how the ApoA-I structure modulates the metabolism and function of HDL. In fact, Apo A-I is essential for the correct assembly, overall stability of HDL and the regulation of HDL metabolism.

1.3.1.2.2 Apolipoprotein A-II (ApoA-II)

ApoA-II is the second most important HDL apolipoprotein and represents approximately 15-20 % of total HDL protein. About half of the HDL particles may contain ApoA-II. ApoA-II circulates as a 17 kDa protein and its synthesis takes place mainly in liver.[110] Like ApoA-I it is also synthesized as a propeptide (100 aa residues) from a gene on chromosome 1 and is posterior cleaved to release 23 aa residues.[111] ApoA-II transcription is upregulated by nuclear receptors such as peroxisomal proliferative-activated receptor-alfa (PPAR-α), retinoid X receptor (RXR) and SREBP-2.[112,113]

ApoA-II circulates as a homodimer of two identical polypeptide chains, each containing 77 aa and linked by a single disulfide bond.[111] The presence of a cysteine residue allows ApoA-II to form heterodimers with other cysteine containing apolipoproteins, such as ApoE and ApoD. Although it has amphipathic properties, ApoA-II is more hydrophobic than ApoA-I.

Functionally, ApoA-II can both increase HDL particle stability and cause conformational changes in ApoA-I on HDL particles by displacing ApoA-I competitively from the HDL surface and thereby reducing lecithin:cholesterol acyltransferase (LCAT) activation.[114,115] Despite the relative abundance of ApoA-II, its role in HDL metabolism and whether it is an anti-atherogenic or pro-atherogenic protein is not completely clear.

1.3.1.2.3 Others apolipoprotein from HDL

The remainder apolipoproteins of HDL comprises the minor peptides ApoA-IV, V, C-I, C-II, C-III, D, E, F, H, J, L-1 and M, which have structural and functional roles as modulators of enzyme activities.

ApoA-IV is a glycoprotein synthesized primarily in the enterocytes of the small intestine during fat absorption and is secreted into circulation on the surface of newly formed chylomicron particles. The *in vitro* and *in vivo* properties of ApoA-IV include a role in the protective functions against inflammatory diseases[116] and atherosclerosis.[117-119]

ApoA-V is predominantly located in TG-rich particles, chylomicrons and VLDL, but also on HDL, and its functions are activation of LPL, inhibition of hepatic production and secretion of TG.[120]

ApoC-I is associated with both HDL and VLDL and can readily exchange between them. ApoC-I is involved in the accumulation of cholesterol ester in nascent HDL via inhibition of CETP and potential activation of LCAT.[121,122] In addition, ApoC-I modulates the interaction of ApoE with VLDL and inhibits the binding of VLDL to the LDL receptor.[123]

In plasma, ApoC-II and C-III are associated with HDL and VLDL and can exchange between their surfaces. Excess ApoC-II has been associated with the increase of TG-rich lipoproteins and modifications in the HDL particle distribution, factors that may boost the risk of CVD.[124,125]

ApoD is mainly associated with HDL. Unlike others, ApoD does not possess a typical apolipoprotein structure and belongs to the lipocalin family, which is a multifunctional family of carrier proteins.[126,127] ApoD has been described in association with various neurological disorders, including Alzheimer's disease, Parkinson's disease and stroke.[126,128]

The major fraction of circulating ApoE is carried by TG-rich lipoproteins in which ApoE mediates their receptor binding, internalization and catabolism. Interestingly, ApoE is the evolutionary precursor of mammalian ApoA-I. Human ApoE exists as three major isoforms, ApoE2, ApoE3 and ApoE4, which differ by aa substitutions at positions 130 and 176. Human ApoE3 preferentially binds to HDL, while ApoE4 preferentially binds to VLDL.[129] ApoE isoforms are associated with different levels of disease's risk, most notably for atherosclerosis[130] and Alzheimer's disease.[131]

ApoF is present in human HDL and LDL, being also known as lipid transfer inhibitor protein due to its ability to inhibit CETP-mediated lipid transfer between

lipoproteins.[132]Human and mouse ApoF overexpression reduce HDL-C levels, improve the clearance of HDL-CE from the plasma and result in HDL particles that are more efficient acceptors of macrophage-derived cholesterol.[133]

ApoH, also known as beta-2-glycoprotein I (β_2GPI) is a multifunctional phospholipid binding protein, which binds particularly cardiolipin but also various types of negative charged substances such as heparin. ApoH may prevent activation of the intrinsic blood coagulation cascade by binding to phospholipid on the surface of damaged cells. It also regulates platelet aggregation by inhibiting the generation of factor Xa or XI[134] and activating both factor XIIa and protein C. ApoH has other physiological roles, that include apoptotic cell clearance,[135] binding to oxidized low-density lipoprotein (oxLDL),[136] and has been found to be the most relevant antigen target in the subset of antibodies towards oxLDL.[137,138]

ApoJ, also called clusterin, presents a structure that allows binding to a wide spectrum of hydrophobic molecules and to specific cell-surface receptors. ApoJ was classified as a functional homologue of the small Heat Shock Proteins (HSP)[139] in many age-related diseases including neurodegeneration, vascular damage, diabetes and tumorigenesis.[140]

ApoL-I is a key functional element of the trypanosome lytic factor of human serum and is associated with HDL.[141] Lack of this protein from mutations in both APOL1 alleles leads to trypanosome infection in humans.[142]

ApoM, first identified and characterized in 1999, is the latest addition to the apolipoprotein family.[143] ApoM is selectively expressed in hepatocytes and in kidney cells. It is mainly found in HDL, but also in VLDL and LDL. Data from knockout (KO) mice shows that ApoM is critical for the formation of HDL, notably pre-β HLD (lipid-poor ApoA-I).[144] Overexpression of ApoM in LDL-receptor-deficient cholesterol fed mice protects against the development of atherosclerosis through a not yet clarified mechanism.[144]

1.3.1.2.4 Paraoxonase 1 and 3 (PON1 and 3)

Human paraoxonases are calcium dependent lactonases and consist in three members PON1, PON2 and PON3. The three PON genes are aligned on the long arm of chromosome 7q21.3-22.1 and are well conserved in mammals sharing 79-95% aa identity and 81-95% identity at the nucleotide level between different species.[145]

The name “PON” reflects the ability of PON1, the first enzyme discovered of the family to catalyze the hydrolysis of the active metabolite of the organophosphate (OP) insecticide

parathion, the paraxon, which is the substrate commonly used to measure paraoxonase activity (EC 3.1.8.1, aryldialkylphosphatase). PON1 is capable of hydrolyzing a broad spectrum of toxic oxon metabolites of OP insecticides, nerve gases as well as a number of aromatic carboxylic acid esters.[146] PON1 also hydrolyzes phenylacetate which corresponds to the arylesterase activity. Although its esterase activity has been known for long, only more recently the natural and /or endogenous subtract for PON1 was found. PON1 is primarily a lactonase[147,148] capable of hydrolyzing a variety of lactones, including certain drugs (e.g. spironolactone, mevastatin, lovastatin, simvastatin and prulifloxacin), endogenous compounds (e.g. lactone metabolites of arachidonic acid, or hcy-thiolactone) and acyl-homoserine lactones, which are quorum sensing signals of pathogenic bacteria.[148,149]

Phylogenetic analysis shows that PON2 is the oldest member of the family followed by PON3 and PON1.[150] Human PON1 is synthesized mainly in the liver and circulates in the bloodstream associated to HDL. Similarly, PON3 is expressed typically by the liver and at low levels by the kidney and is also associated with HDL in circulation albeit at much lower levels than PON1.[151] In contrast PON2 is not detectable in serum associated with HDL although it is ubiquitous expressed and found in many tissues and cells including macrophages, heart, brain, liver, kidney, lung, placenta, small intestine, spleen, stomach and testis.[152]

Current findings suggest that the name PON is in fact a misnomer, as the capacity of hydrolase OP (paraoxonase activity) is almost exclusive of PON1. All three PONs have arylesterase activity, although very low activity with the exception of PON1. Nevertheless PON1, PON2, and PON3 share the lactonase activity with overlapping but also distinct substrate specificity.[153] Dihydrocoumarin, long chain fatty acid lactones and acyl-homoserine are hydrolyzed by all three PONs and likely represent their natural subtracts.[154] The three members of the family seem to have an important role in the maintenance of a low oxidative state in plasma by protecting against or reversing HDL and LDL oxidation.[155] Despite a growing interest on PON2 and PON3, PON1 still by far the best studied member of the family.

PON1 gene sequence identified almost 200 polymorphic sites on the coding and promoter regions. The two most studied and common coding polymorphisms are at position 192 leading to a glutamine – arginine substitution (Q192R) and at position 55 the leucine to methionine substitution (L55M). These polymorphism exhibits an exogenous substrate-dependent effect on enzyme activity. For instance, subjects with the RR genotype have

significantly higher paraoxonase activity towards paraoxon than subjects with QQ genotype, but hydrolyze diazoxon, soman, and sarin less rapidly.[156,157] Both isoforms of the enzyme hydrolyze phenyl acetate at approximately the same rate and do not affect PON1 protein concentration.[148] Regarding the other polymorphism at position 55 it seems to affect enzyme quantity, rather than specific activity or substrate preference although this is not yet fully clarified.[158] The binding affinity and stability of PON1 on HDL is affected by the PON1 polymorphisms.[159]

The association between PON1 polymorphisms and CHD remains controversial, few studies showed a positive association between PON1 192 RR genotype and CHD whereas many others failed to confirm this. Recently was performed a meta-analysis on the relation between PON1 polymorphism and CHD which included 88 studies published before August 2010, with a total of 24,702 CHD cases and 38,232 controls.[160] This study reported a weak overall association with the PONQ192R with a doubtful relevance since there was no significant association amongst the larger studies included in the analysis, thereby replicating the results of past.[161-163] However low PON1 activity seems to be associated with atherosclerosis independently of the PON1 polymorphism and appears to be a better predictor of CVD development than the PON1 genotype.[157,164,165]

PON1 activity and/or level of expression are regulated by several exogenous factors such as life –style, dietary factors and age.[166,167] PON1 activity increases with the intake of lipid lowering drugs, antidiabetic and hormone-replacement drugs.[168]

Some of recent studies regarding the PON1 lactonase activity are focused in its capacity to hydrolyse Hcy - thiolactones. Elevated blood levels of Hcy (an intermediate metabolite in methionine biosynthesis) is a known risk factor for atherosclerosis and is associated with a moderately risk of CVD.[169] The metabolic conversion of Hcy to Hcy - thiolactone is a mistake reaction of the methionyl-tRNA synthetase which by an editing error selected Hcy instead of methionine.[170] The Hcy-thiolactone formed is a reactive metabolite that has the ability to establish isopeptide bonds with protein lysine residues that cause protein N-homocysteinylation[171] which has detrimental effects on protein structure and function leading to severe pathophysiological consequences including activation of the immune response[172] and enhanced thrombosis.[173]

Several studies show that Hcy-thiolactonase activity of PON1 inhibits the Hcy-thiolactone formation and consequently N-homocysteinylation of proteins,[147,174,175] so in

addition to the anti-oxidant and anti-inflammatory functions of peroxidase and esterase activity of PON1, the thiolactonase activity also contributes significantly for its cardioprotective function.

1.3.1.2.5 Lecithin-cholesterol acyltransferase (LCAT)

LCAT (*EC 2.3.1.43*) catalyzes the esterification of cholesterol to CE by transferring the fatty acid from the second position on the glycerol backbone of lecithin or phosphatidylethanolamine to the free hydroxyl group on the first ring of cholesterol.[176] The generated CE is very hydrophobic molecules that migrate into the inner core of the particle, creating a pool of so-called "neutral lipid," which is completely encapsulated by a surface coat of phospholipid, free cholesterol, and apolipoprotein. Cholesterol esterification by LCAT occurs predominantly in HDL, but can also take place in ApoB containing particles.

LCAT plays an important role in RCT as the majority of tissue-derived cholesterol is delivered to the liver as CE and only a small amount as free cholesterol. CE trapped in the HDL core cannot be spontaneously exchanged with other lipid molecules until it is removed from the circulation by the liver.[177] In parallel, LCAT promotes cholesterol efflux from peripheral cells, acting via the elevation of the concentration gradient for unesterified cholesterol between cell membranes and HDL.

LCAT is a 63 kDa protein synthesized mainly in the liver and, to a lesser extent, in the brain and testes. Mature LCAT contains 416 aa residues and is heavily glycosylated.

In plasma LCAT is closely associated with ApoD. ApoA-I mediates the binding of LCAT to HDL and its activation. Others apolipoproteins, including ApoA-IV, C-I, E, are also capable of activating LCAT, although less potently compared to ApoA-I.

LCAT has been shown to directly hydrolyse oxidized polar phospholipids and also prevent the accumulation of oxidized lipids in LDL.[178,179] However aldehydes that are generated during lipid peroxidation can modify HDL structure and inhibit LCAT activity.[180,181] LCAT activity is also decreased in HDL isolated from diabetic subjects (and in *in vitro* glycated HDL), due to the glycation of lysine residues in ApoA-I.[182,183] Furthermore, the displacement of ApoA-I by serum amyloid A (SAA) during acute phase reaction inhibits LCAT activity.[184]

1.3.1.2.6 Cholesterol ester transfer protein (CETP)

CETP is a plasma lipid transfer protein, primarily expressed by the liver and adipose tissue. The mature CETP contains 476 aa residues and multiple glycosylation sites. In

circulation, this hydrophobic 74 kDa glycoprotein facilitates the bidirectional transfer of CE and TG between mature spherical HDL and ApoB containing lipoproteins. Specifically, CETP transfers CE from HDL to ApoB containing lipoproteins, such as VLDL, IDL and LDL, in exchange for TG (Figure 5).[185]

Its substrate specificity is regulated by lipid transfer inhibitor protein (or ApoF).[132] These processes deplete the HDL core of CE and enrich them with TG. Therefore CETP promotes HDL-mediated cholesterol efflux from peripheral cells via elevation of the concentration gradient for unesterified cholesterol between cell membranes and HDL. HDL-derived CE that accumulate in ApoB containing lipoproteins can be subsequently delivered to the liver through the hepatic LDL receptor, as described before (sub-chapter 1.2.2). Thus, in addition to the important role in RCT, CETP actions are equally implicated in the intravascular remodelling of lipoproteins, which favours HDL particle recycling and thus the formation of lipid-free/lipid-poor ApoA-I, thereby leading to enhanced cellular free cholesterol efflux and plasma LCAT activity.

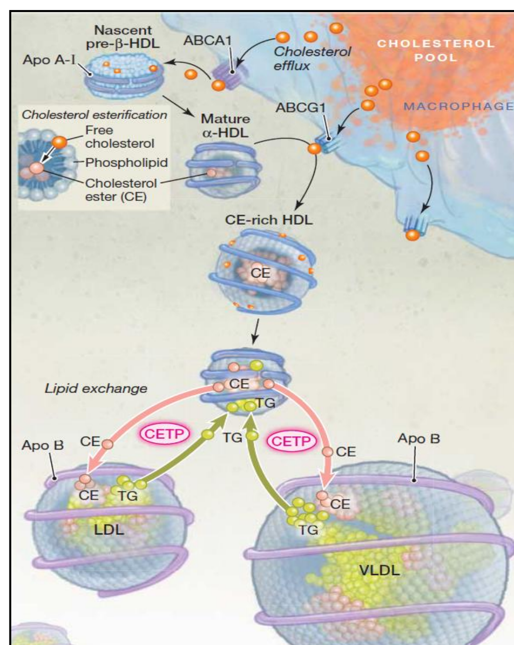


Figure 5. Cholesterol ester transfer protein (CETP)-mediated lipid exchange between lipoproteins. From Nicholls et al, 2011.[186]

Such actions are synonymous to an anti-atherogenic role of CETP. A second major CETP mediated pro-atherogenic pathway takes place in states of moderate to marked hypertriglyceridaemia, in which CETP drives an enhanced transfer of TG from VLDL to HDL, leading to TG enrichment of HDL with anomalous intravascular metabolism involving HL mediated hydrolysis of HDL phospholipids and TG, with a consequent reduction of particle

size, formation of small dense HDL and dissociation of lipid-poor ApoA-I. This lipid-poor ApoA-I instead of acting as an acceptor of cholesterol from pheriferic tissues can be removed and lost irreversibly from plasma due to accelerated renal catabolism.[187-189] Therefore CETP may exert both pro-atherogenic and anti-atherogenic actions, the balance between them clearly depending primarily on the metabolic context.

The importance of CETP in HDL metabolism is demonstrated by the discovery of individuals who have loss-of-function mutations in both alleles of the CETP gene. These subjects, found almost exclusively in Japan, have extremely elevated levels of HDL-C.[190,191] In addition, their HDL is exceptionally large, and the turnover of ApoA-I is substantially reduced.[192]

1.3.1.2.7 Other HDL components

Platelet activating factor acetylhydrolase (PAF-AH, EC 3.1.1.47) whose plasma form is known as lipoprotein-associated phospholipase A2 (Lp-PLA2), is a calcium-independent enzyme that degrades the short *sn*-2 residue of PAF to the biologically inactive lyso-PAF, as well as pro-inflammatory oxidized short-chain phospholipids and esterified isoprostanes.[193] This action of Lp-PLA2 indicates that it may be a potent anti-inflammatory enzyme. However, Lp-PLA2 also generates lysophosphatidylcholine and bioactive oxidized non-esterified fatty acids, two key pro-inflammatory mediators implicated in atherosclerosis.[194] Thus, Lp-PLA2 could play both pro-atherogenic and anti-atherogenic role depending on which lipoprotein the enzyme is associated in plasma.[195]

Other minor constituent of HDL are: 1) the glutathione peroxidases isoenzyme 3 (GPx3) that may reduce the oxidative damage by catalyzing the glutathione dependent reduction of lipid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water.[196] 2) the phospholipid transfer protein (PLTP) that aids in the transfer of phospholipids between HDL and VLDL, as well as between different HDL particles.[197] 3) the sphingosine-1-phosphate (S1P) that is a bioactive lipid involved in various cellular processes such as cell proliferation, motility, apoptosis, angiogenesis, wound healing and immune response.[198]

1.3.2 Atheroprotective functions of HDL

Recently, the interest in HDL has spiked, with the recognition of its multiple anti-atherogenic properties. The best known anti-atherogenic function of HDL is the capacity to promote cellular cholesterol efflux from peripheral cells and deliver cholesterol to the liver

for excretion, thereby playing a key role in RCT. However the anti-atherogenic roles of HDL goes beyond RCT and involve anti-oxidant, anti-inflammatory and antithrombotic properties, as well as cytoprotective, vasodilatory, anti-infectious and antidiabetic activities (Figure 6).[199]

It is believed that the varied physiological functions of HDL reflect, the wide variety of proteins carried by HDL and the heterogeneity of HDL complexes; indeed, specific HDL associated functions appear to be mediated by one or more distinct proteins potentially acting synergistically. Nearly all HDL subpopulation display multiple atheroprotective functions, many of these activities are particularly effective in small, dense, protein-rich HDL.[200] Not only the protein content of HDL may influence its functions, the lipid composition of HDL may interfere with the association of the different proteins at the HDL surface.

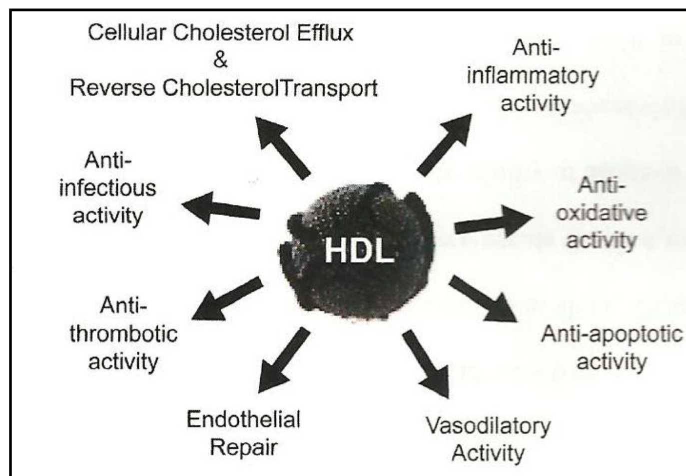


Figure 6. Summary of the key anti-atherogenic properties attributed to high density lipoprotein (HDL). From Yamashita et al, 2010.[199]

1.3.2.1 Reverse cholesterol transport (RCT)

Promotion of cholesterol efflux from macrophages on the arterial wall for excretion into bile constituents, completing the RCT pathway is thought to be one of the most important mechanisms by which HDL protects against atherosclerosis (Figure 7).

ApoA-I is synthesized by the liver and released into the plasma as relatively free from lipid, known as nascent HDL. Small nascent HDL are unstable and rapidly receives free cholesterol and phospholipids from tissues by a unidirectional active transport process that is mediated by a family of transmembrane adenosine triphosphate-binding cassette proteins (ATP-binding proteins). The best characterized member of this family, is adenosine triphosphate-binding cassette transporter A1 (ABCA1) which preferentially efflux cholesterol to lipid deplete or free forms of ApoA-I producing discoid HDL particles. Such particles

further acquire cellular lipids via pathways mediated by ABCA1 and others ATP-binding proteins as the ABCG1 and ABCG4 which preferentially binds to mature spherical HDL particles.[201]

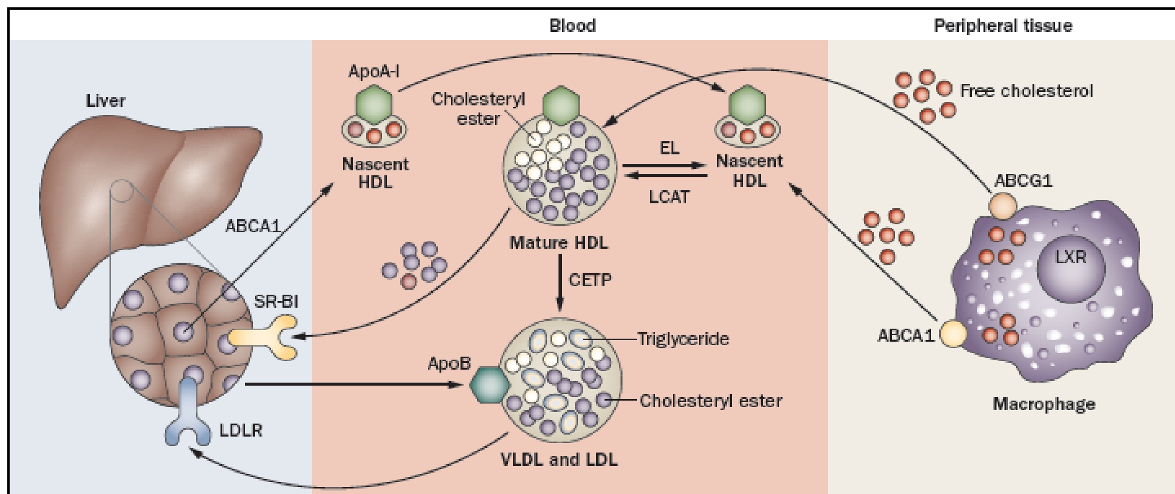


Figure 7. HDL metabolism and reverse cholesterol transport (RCT). The liver secretes lipid-poor apolipoprotein A-I (ApoA-I), which quickly acquires cholesterol via the hepatocytes ATP-binding cassette sub-family member 1 transporter (ABCA1). Lipid-poor ApoA-I also promotes the efflux of free cholesterol from macrophages via ABCA1. Lecithin cholesterol acyltransferase (LCAT) esterifies free cholesterol to cholesteryl esters (CE) to form mature HDL, which promotes cholesterol efflux from macrophages via the ABCG1 transporter, as well as from other peripheral tissues by processes not fully defined. Mature HDL can transfer its cholesterol to the liver directly via scavenger receptor class B type I (SR-BI) or indirectly via cholesterol ester transfer protein (CETP) mediated transfer to ApoB-containing lipoproteins, with subsequent uptake by the liver via the LDL receptor. Hepatic cholesterol can be excreted directly into the bile as cholesterol or after conversion to bile acids and, unless reabsorbed by the intestine, is ultimately excreted in the faeces. HDL can be remodelled by lipases such as hepatic lipase (HL) and endothelial lipase (EL), which hydrolyze HDL triglycerides and phospholipids, respectively. From Duffy et al, 2009.[202]

Excess of cholesterol can also be removed from cells by scavenger receptor class B type I (SR-BI) that promotes an ATP-independent bidirectional cholesterol flux between cellular membranes and large HDL particles according to concentration gradients.[203]

Within the HDL particle, unesterified cholesterol can be esterified to CE by the addition of fatty acid to the cholesterol molecule through the action of the HDL associated LCAT, hereafter this newly formed CE migrates into the hydrophobic core of immature discodial HDL turning it into a mature and spherical HDL.[204] Since the particles on the surface remains relatively deplete of cholesterol the gradient driving efflux from cells to the HDL particle is maintained.

Ultimately, HDL must deliver the cholesterol acquired via transporters to the liver for bile excretion as free-cholesterol or bile acids. Cholesterol transported by HDL can arrive to the hepatocyte by several ways. Perhaps the most common is via SR-BI expressed in the hepatocytes. Unlike what happens with LDL when it binds to its receptor the binding of HDL

to SR-BI does not result in the internalization of the complex formed. In this case the SR-BI mediates the selective translocation of the CE of HDL, but not protein, by forming a hydrophobic channel through the plasma membrane designate caveolae while the HDL and SR-BI remain on the plasma membrane.[205] Thus, HDL particles are quickly recycled and returned to the cell surface as a smaller particle (lipid-poor ApoA-I) ready to acquire more free cholesterol and continue the loading process to larger particles.[206]

HDL particles can also be removed from the circulation by holoparticle HDL receptors. Both liver and kidney can rapidly take up holo-HDL particles, including ApoA-I, by endocytosis and can be subsequently transferred to multivesicular bodies and, to a minor degree to lysosomes for degradation.[207] In the last thirty years have been identified several potentially candidates leading to HDL holoparticle or ApoA-I uptake and degradation, including cubilin, megalin, ApoA-I binding protein (AI-BP), two other membrane proteins named HB1 and HB2, also high density lipoprotein binding protein (HBP) and more recently the ectopic β-chain of ATP synthase.[73]

Alternatively, an indirect pathway can occur and CE from HDL is transferred to ApoB containing lipoproteins such VLDL, IDL and LDL in exchange for TG through the action of CETP.[208] PLTP can transfer phospholipids from ApoB containing lipoproteins to HDL.[197] Following transfer to ApoB containing lipoproteins CE are either taken up by the liver via LDL receptor or delivered to cells in the periphery. In healthy, normolipidemic individuals such indirect RCT pathway to delivery CE to the liver accounts for 70 % of CE removal from peripheral tissues.[209]

1.3.2.2 Anti-oxidant function of HDL

Oxidative stress has a key role in initiation and propagation of atherosclerosis and its associated complications.[210] Oxidative stress results from an imbalance between increased generation of intracellular reactive oxygen species (ROS) and the decreased ability of endogenous antioxidant systems to scavenge them. Molecules generated during oxidative stress may damage the normal structure / functions of proteins, lipids, carbohydrates, nucleic acids and thus affect diverse cellular processes like membrane function, enzyme activity and DNA replication. Enhanced production of ROS has been attributed to increased activation of enzymes such as myeloperoxidase (MPO), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, lipoxygenase and cyclooxygenase (COX), as

well as dysfunction of the mitochondrial respiratory chain. ROS may oxidize phospholipids within LDL. OxLDL is a powerful chemoattractant for monocytes, and the phagocytosis of oxLDL by monocytes is a critical step for transformation of monocytes into foam cells.[211]

The anti-oxidant functions of HDL are explained by its content of anti-oxidants, ApoA-I and several enzymes such as PON, PAF-AH and GPx3 which directly or indirectly prevent LDL oxidation.[212-216] Furthermore, HDL is cytoprotective against apoptosis of vascular cells elicited by oxLDL.[217]

The importance of PON's anti-oxidant effect has been demonstrated in several studies: PON1 was highly effective in preventing LDL oxidation,[218,219] moreover PON1 inhibits macrophage cholesterol biosynthesis by attenuating oxLDL uptake by macrophages[220] and also stimulates HDL-mediated cholesterol efflux from macrophages.[221] Anti-inflammatory actions of PON1 has also been reported as it decrease monocyte chemotaxis and adhesion to endothelial cells[222] Furthermore, transgenic animals deficient in PON1 were more susceptible to atherosclerosis development,[223] while those that overexpressed PON1 had less atherosclerosis.[224]

With the finding that the major activity of PON's is a calcium dependent lactonase and that the affinity of PON1 for lipid hydroperoxides is several orders of magnitude lower than its affinity for lactones, the established anti-atherosclerotic properties of PON1 may also involve its major activity as lactonase. In particular, by hydrolyzing Hcy-thiolactones and protecting against the pro-atherogenic modifications of proteins caused by them.[225]

In addition to ApoA-I, other HDL apolipoproteins display antioxidative activity such as ApoE[226], ApoJ[227] and ApoA-IV[228]. ApoE possesses distinct antioxidative properties and can promote regression of atherosclerosis independently of the reduction in plasma cholesterol levels.[229] Moreover, HDL particles also contain small amounts of lipophilic anti-oxidants, mainly tocopherols, which may supply a minor input to antioxidative properties of HDL.[230]

1.3.2.3 Anti-inflammatory function of HDL

HDL display multiple anti-inflammatory properties which together may significantly contribute to its anti-atherosclerotic properties.[231-233]

HDL decreases the cytokine-induced adhesion molecule expression on endothelial cells and inhibits monocyte adhesion to the endothelium. *In vitro* HDL particles potently inhibit the expression of VCAM-1, intercellular adhesion molecule-1 (ICAM-1) and E-selectin

induced in endothelial cells by tumor necrosis factor-alpha (TNF-α), interleukin -1 (IL-1), endotoxin or C-reactive protein (CRP) .[213, 234-236]

These affects may be accomplished by HDL inhibition of factor nuclear kappa B (NF-κB) activation induced by oxLDL and TNF-α .[214]

Furthermore HDL can inhibit monocyte activation via reducing the expression of chemokine C-C motif ligand 2 (CCL2), CCL5 and chemokine C-X3-C motif ligand 1 (CX3CR1), as well as chemokine receptors like C-C chemokine receptor type 2 (CCR2) and CX3CR1.[237]

In vivo, infusions of reconstituted HDL (rHDL) inhibit adhesion molecules expression across arterial endothelium in a rabbit model of acute arterial inflammation.[238] Intravenous infusions of rHDL (ApoA-I/phosphatidylcholine) decreases VCAM-1 expression in atherosclerotic plaques obtained from patients with peripheral vascular disease in a placebo-controlled trial.[236,239]

HDL-associated ApoA-I displays inhibitory activity towards contact-mediated activation of human monocytes by stimulated T-lymphocytes.[240] As direct cell-cell contact with stimulated T lymphocytes is a strong inducer of cytokine production in monocytes, HDL specifically inhibits the production of some pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-8, CCL3 and CCL4) induced by T-cell contact.[241]

In addition ApoA-I inhibits oxLDL-induced monocyte chemotaxis and significantly decreases lipopolysaccharide (LPS)-mediated monocyte chemotactic protein-1 (MCP-1), sL-selectin, sICAM-1 and sVCAM-1 release from THP-1 cells.[242]

Apart from turning into foam cells a significant proportion of blood monocytes differentiated into migratory dendritic cells (DCs). These monocyte-derived DCs may migrate via the afferent lymph and have a considerable role in the removal of dying cells from atherosclerotic lesions, which could be a solution to solve the local inflammation. Nevertheless, this emigratory process is impaired in atherosclerosis[243] as a consequence of inhibitory signals generated by PAF or oxLDL that acts as a PAF mimetic. [244]

These multiple anti-inflammatory properties of HDL suggest that several action mechanisms may be operative at one time: the cellular lipid efflux mediated by ABCA1 and ABCG1 is a mechanistic basis that explains the capacity of HDL to decrease adhesion molecule expression and to inhibit monocyte and neutrophil activation, probably due to the rapid depletion of cholesterol from cell membranes.[245,246] The removal of cellular cholesterol by HDL also results in changes in the expression of genes involved in the regulation of the

inflammatory response, as the down-regulation the inflammatory phenotype of macrophage with consequent attenuation of signalling via toll-like receptor (TLR) 4.[247]

On the other hand the ability of HDL to inhibit adhesion molecule expression may be related to the presence of several protein and lipid components, which include ApoA-I, ApoA-II, ApoA-IV and HDL-associated lysosphingolipids, such as S1P.[119,235,248]

Transforming growth factor beta (TGF-β) possesses several anti-inflammatory properties and stabilizes the atherosclerotic plaque.[249] HDL induces TGF-β2 expression *in vitro* and *in vivo* via phosphoinositide 3-kinase (PI3K)/Akt activation, running as another important mechanism by which HDL may exert protective effects on endothelial cells and vascular wall.[250]

Modulation of ILs can be equally involved in the effects of HDL on inflammatory cascade. In fact, HDL attenuates the expression of pro-inflammatory cytokine, such as IL-1β and IL-6,[239,251] but increases the production of anti-inflammatory cytokines as IL-10.[252]

1.3.2.4 Vasodilatory function of HDL

One major indicator of endothelial dysfunction in the development, progression, and clinical complications of atherosclerosis is the decreased endothelial $NO^{\bullet}$ production and bioavailability, as result of enhanced inactivation of $NO^{\bullet}$ by superoxide anion ($O_2^{\bullet -}$) [253,254] This leads to increased neutrophil adherence to the endothelium, enhanced smooth muscle cells (SMCs) proliferation and platelet aggregation and adhesion.

HDL particles display potent vasodilatory activity, which reflects their capacity to modify endothelial nitric oxide synthase (eNOS) expression as well as its activity and the stimulation of endothelial $NO^{\bullet}$ production *in vitro* and *in vivo.*[255,256]

Moreover, administration of rHDL has been shown to improve endothelial function in subjects with hypercholesterolemia and in subjects with isolated low HDL due to heterozygous loss-of-function mutations in the ABCA1 gene locus.[257,258]

Several different mechanisms have been proposed to account for the endothelial $NO^{\bullet}$ stimulating capacity of HDL (Figure 8).

Endothelial NOS is localized in caveolae, specialized microdomains prevalent on the plasma membrane of endothelial cells that contain cholesterol, sphingomyelin, glycosphingolipids, phospholipids and membrane proteins. The structure of caveolae depends on the type and amount of cholesterol associated with the domain and a sufficient

level of cholesterol is required to maintain the invaginated structure of the caveolae and its association with eNOS. Lipoproteins may modulate eNOS activity by interacting with the receptors found in caveolae such as SR-BI, CD36 and the caveolae protein, caveolin-1. It is known for long that oxLDL depletes caveolae of cholesterol, via binding to CD36 leading to the intracellular translocation of eNOS and a reduction in the capacity to activate eNOS.[259] On the contrary, HDL binding to SR-BI maintain the concentration of caveola-associated cholesterol by promoting the uptake of CE. Therefore oxLDL-induced depletion of caveola cholesterol is prevented and the subcellular location of eNOS is mantained.[260]

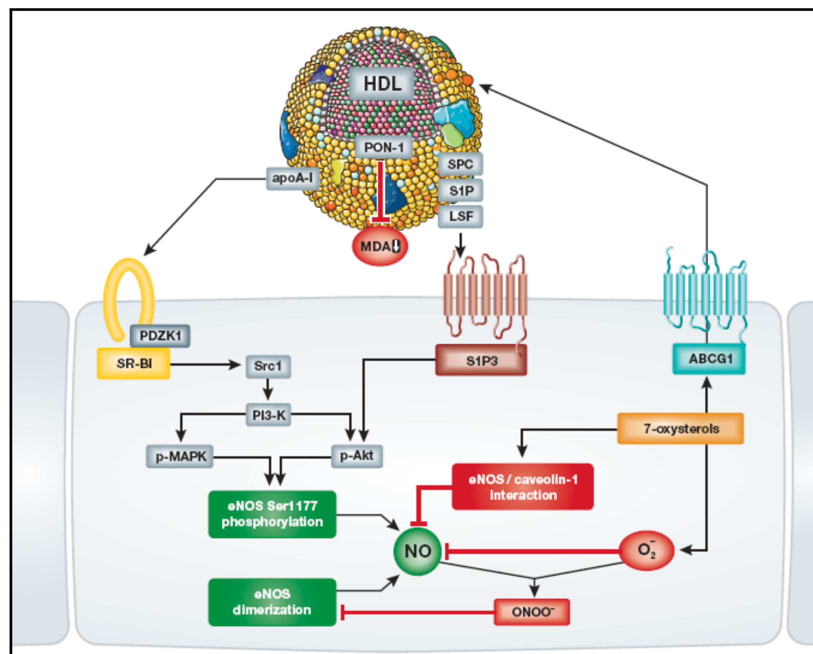


Figure 8. Major pathways involved in vasodilatory activity of HDL. HDL enhances nitric oxide (NO•) production by endothelial NO synthase (eNOS) phosphorylation at serine residue 1177 via binding of ApoA-I to SR-BI and binding of HDL-associated lysophospholipids to the S1P3 receptor. Also, HDL mediated efflux of 7-oxysterols through endothelial ABCG1 has been observed to inhibit the interaction between eNOS and caveolin, and to prevent the loss of eNOS dimerization induced by ROS in the endothelium. From Besler C et al, 2012.[261]

Yuhanna et al[262] have shown that HDL directly stimulate eNOS mediated NO• production through the biding of ApoA-I to SR-BI in endothelial cells.

Mechanistically, the binding of HDL to SR-BI leads to a tyrosine kinase Src-mediated activation of PI3K, which in turn activates Akt and the mitogen-activated protein kinases (MAPK) /extracellular signal-regulated kinase pathway.[255,263] This activation of endothelial Akt has been shown to stimulate phosphorylation of eNOS at serine residue 1177, recognized as an key regulatory mechanism that leads to eNOS activation.[264]

In contrast, the mechanism through which the MAPK/extracellular signal-regulated kinase pathway activates eNOS in endothelial cells stimulated with HDL remains poorly understood. Nevertheless, several lysophospholipids found in HDL (i.e. sphingosylphosphorylcholine, S1P, lysosulfatide) can via Akt-mediated activation of eNOS independently release $NO^{\bullet}$ through binding of lysophospholipid receptor S1P3 that is expressed in endothelial cells.[263]

Another pathway whereby HDL may maintain endothelial cell $NO^{\bullet}$ production and availability is mediated by ABCG1 and involves cholesterol efflux.[265] Studies with cholesterol-fed mice suggested that HDL-mediated efflux of cholesterol and 7-oxysterols (a dietary oxysterol) via endothelial ABCG1 improving the formation of active eNOS dimers and results in decreased ROS production.[265] In support of these data, vascular deficiency of ABCG1 impairs eNOS-mediated relaxation and accelarates atherosclerosis in LDL receptor-deficient mice.[266] Furthermore, HDL-mediated cholesterol efflux via ABCG1 reduced the inhibitory interaction of eNOS with caveolin-1 induced by cholesterol loading resulting in increased eNOS activity.[267]

Recently, Besler et al[256] suggested that inhibition of PON1 in HDL from healthy subjects impaired the capacity of HDL to stimulate Akt/eNOS phosphorylation, endothelial $NO^{\bullet}$ production and $NO^{\bullet}$-dependent vasodilatation. Consistent with these findings, HDL isolated from PON1-deficient mice failed to stimulate endothelial $NO^{\bullet}$ production. These data suggest a role of HDL-associated PON1 activity in maintaining the endothelial atheroprotective effects of HDL, at least in part by preventing formation of the lipid peroxidation product malondialdehyde (MDA) rather than directly degrading MDA. Furthermore, inhibition of PON1 prevented the protective effects of HDL from healthy subjects on TNF-α–stimulated endothelial VCAM-1 expression, endothelial monocyte adhesion and endothelial repair, suggesting that the capacity of HDL to stimulate endothelial $NO^{\bullet}$ production is important for the vasodilatory and anti-inflammatory effects of HDL.

HDL can also stimulate the production of PGI2, which possesses potent vasorelaxing activity and therefore being able to modify thrombosis as well as other intravascular events.[268,269] Furthermore, PGI2 acts synergistically with $NO^{\bullet}$ to induce vascular smooth muscle relaxation, inhibit platelet activation and adhesion and diminish the release of growth factors that stimulate the local proliferation of vascular SMCs.

The effect of HDL on prostacyclin production in the endothelium occurs both by the provision of arachidonate[268,270] and upregulation of COX-2 expression.[271,269] Several different mechanisms have been proposed to account for the HDL mediated COX-2 expression and PGI2 release. HDL induces the phosphorylation of p38 MAPK, which is implicated in the activation of cAMP response element-binding protein (CREB) in endothelium[269] and mimics the mechanism that occurs in vascular smooth muscle.[272] Moreover, the SR-B1 mediated PI3K–Akt–eNOS signalling pathway plays a role in HDL induced PGI2 and COX-2 production in endothelial cells.[273] Also, oxidized steroids may increase COX-2 expression through the PI3K–Akt–eNOS pathway in human umbilical vein endothelial cells (HUVECs).[274]

In addition, HDL has a beneficial effect not only on endothelium, but also on endothelial progenitor cells (EPCs).[275-276] EPCs mainly reside in bone marrow are mobilized into circulation in response to various stimuli of angiogenesis. In the circulation, EPCs express surface CD34, CD133 and KDR (VEGFR-2) and may go to the site of injured endothelial cells and differentiate into mature and healthy endothelial cells to maintain the integrity of the endothelial monolayer.[277] Numerical and functional impairment of EPCs contribute to endothelial dysfunction and the associated increase in cardiovascular risk.[278] A direct link between HDL and EPCs was demonstrated with rHDL infusions in ApoE deficient mice which nearly doubled the number of EPCs, effectively promoted the EPCs-mediated repair of damaged endothelium[275] and enhanced ischemia-induced angiogenesis[279] *in vivo* through the stimulation of differentiation of EPCs. Infusions of rHDL also increased circulating EPCs in patients with type 2 diabetes.[276] HDL prevents the apoptosis of EPCs by activating eNOS and inhibiting caspase 3 *in vitro*.[280] Both ApoA-I, possibly through interaction with SR-BI,[281] and S1P through S1P3 receptor[282] have the ability to stimulate EPCs and promote neovascularization. More recently, was demonstrated that PI3K/Akt-dependent cyclin D1 activation plays an essential role in HDL-induced EPCs proliferation, migration and angiogenesis.[283]

1.3.2.5 Antithrombotic function of HDL

Platelets are implicated in the initiation, progression and rupture of atherosclerotic lesions (Figure 9).[284] Arterial thrombus formation is determined by the balance between prothrombotic mediators, like tissue factor (TF) and plasmogen activator inhibitor-1 (PAI-1),

and antithrombotic such as tissue factor pathway inhibitor (TFPI) and tissue plasmogen activator (tPA).[285]

Dyslipidemia is frequently associated with increased platelet reactivity and thrombogenic potential.[286]

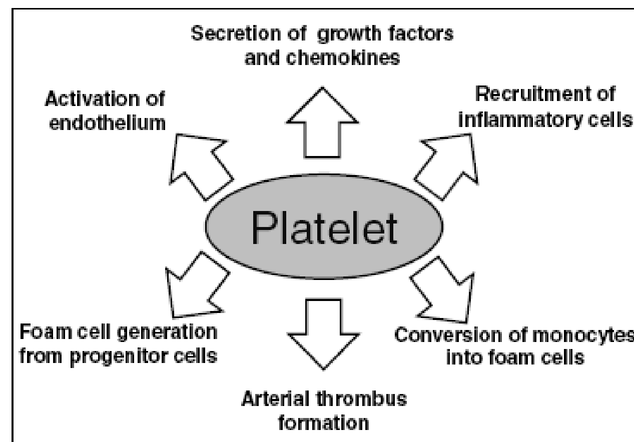


Figure 9. Contribution of platelets to the initiation and the progression of atherosclerosis. From Nofer et al 2010 [284]

HDL exerts multiple antithrombotic effects and thereby counteracts the development of atherothrombotic vascular disease.[284] In fact, infusion of rHDL particles significantly mitigates *ex vivo* platelet aggregation response to multiple agonists activation, in subjects with type 2 diabetes characterized by enhanced platelet aggregation.[287] The *in vitro* antithrombotic activities of HDL on platelets involves a dose-dependent inhibitory action by glycoprotein IIb/IIIa on agonist-stimulated platelet aggregation (such as thrombin, collagen, ADP, adrenaline, oxLDL and arachidonic acid) [288,289] and on thrombin-induced binding of fibrinogen, which is a pre-requisite of platelet aggregation.[290] In addition, HDL-mediates inhibition of thrombin- and ADP-stimulated secretion of alpha- and dense granules as well as the liberation of thromboxane A2 (TxA2) and 12-hydroxyeicosatetraenoic acid (12-HETE). Furthermore, HDL can decrease platelet aggregation as a result of enhanced production of $NO^{\bullet}$.[284]

Blood coagulation involves a series of intrinsic or extrinsic pathways that occurs through the formation of enzymatic protein complexes that assemble on the surface of anionic phospholipids. Although these pathways are initiated by distinct mechanisms, the two converge on a common pathway that leads to fibrin clot formation. HDL inhibits factors that promote blood coagulation, including tissue factors and factors X, Va and VIIIa.[291,292] HDL also has been shown to contain a TFPI,[293] which would turn HDL less thrombogenic, and

thrombin that is generated on the surface of HDL might be 20 times less efficient than when formed on the surface of TG-rich lipoproteins.

Moreover, ApoA-I neutralize the procoagulant properties of anionic phospholipids, which lose their capacity to mediate activation of prothrombin by factor Xa in the presence of factor Va and unable to support binding of factor Va.[294]

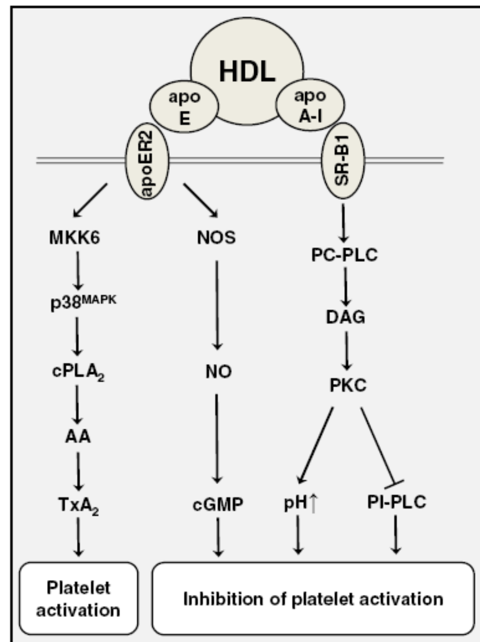


Figure 10. Intracellular signalling cascate triggered by the interaction of HDL with the platelet receptors SR-B1 and ApoER2. SR-B1-mediated interaction prompts the activation of protein kinase C (PKC), which leads to cytoplasmic alkalization and inhibits activity of phosphatidylinositol specific phospholipase C (PI-PLC). ApoER2-mediated interaction induces activation of nitric oxide synthase (NOS) and generation of nitric oxide ($NO^{\bullet}$), which subsequently stimulates the production of cyclic GMP (cGMP). ApoER2 may also induce a signalling cascade encompassing protein kinases MKK3/6 and mitogen activated protein kinase p38 (p38MAPK) as well as phospholipase A2 (PLA2), arachidonic acid (AA), and thromboxane A2 (TxA2). From Nofer et al 2010.[284]

1.3.2.6 Anti-apoptotic function of HDL

In addition to the functions already described HDL also protects macrophages and endothelial cells from apoptosis[215] and from the cytotoxic effects of numerous agents, such as oxLDL,[295] chylomicron remants,[296] TNF-α[297] and protein of complement system.[298]

The most important intracellular mechanisms underlying the anti-apoptotic actions of HDL include: (i) cellular efflux of oxidized cholesterol, mainly of 7-ketocholesterol, mediated by ABCG1;[299] (ii) decreased intracellular generation of ROS,[297] suggesting that cytoprotection can be related to the intracellular anti-oxidant properties of HDL; (iii) preservation of mitochondrial integrity resulting in the inhibition of release of cytochrome c and apotosis-inducing factor from mitochondria followed by the abrogation of caspase cascade activation;[215] (iv) stimulation of $NO^{\bullet}$ synthesis by HDL via interaction with SR-BI and

stimulation of Akt pathway in a PI3K-dependent manner,[255] which is essential for the vasodilatory effect of HDL and (v) stimulation of endothelial cell migration by induction of intracellular signalling via S1P receptors.[300]

1.3.2.7 Other functions of HDL

Furthermore, HDL also has mitogenic activity[301] and can potentiate the beneficial effects of growth factor (EGFR, PDGF, EGF, IGF). HDL induced proliferation of SMCs can strengthen the fibrous cap of the atherosclerotic plaque and improve plaque stability.

The cytoprotective capacity of HDL is not limited to arterial wall cells and appears to represent a broad physiologic phenomenon. HDL modulates the survival of both human and murine β-cells in isolated islets by decreasing basal as well as IL-1β and glucose-induced apoptosis.[302] This study shown that inducible NOS (iNOS), which mediates the pro-apoptotic effects of glucose and cytokines and the death receptor Fas is down-regulated by HDL.

Recent data, suggest that low HDL-C may play a causative role in the development of insulin resistance.[303] It has been shown that HDL may improve glucose metabolism by multiple mechanisms which include enhanced insulin secretion by β-cells as a result of improved cellular cholesterol homeostasis (Figure 11).[303-305]

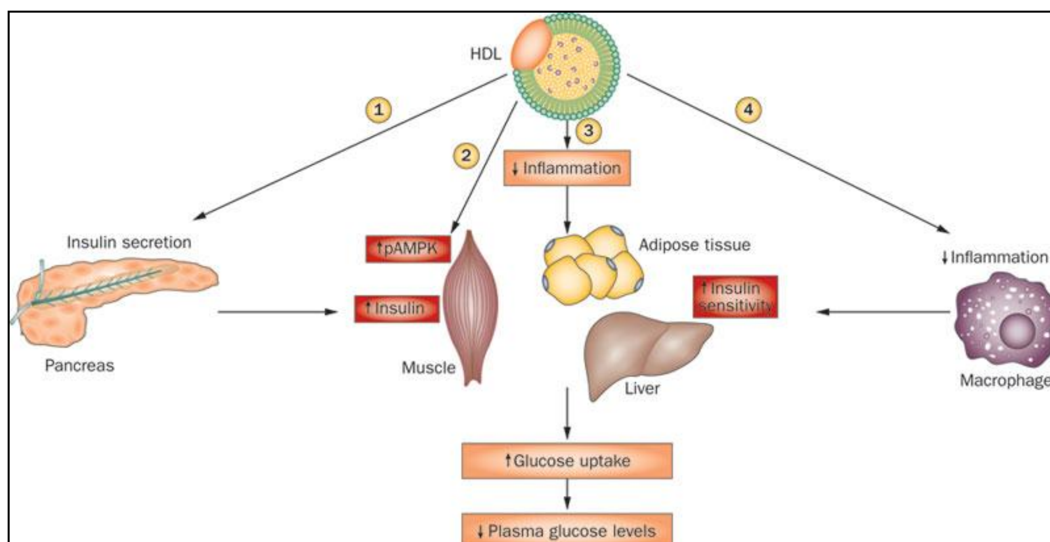


Figure 11. HDL and glucose homeostasis. HDL might regulate the insulin secretion (1), increasing glucose uptake into skeletal muscle (and presumably other peripheral tissues) through non-insulin-dependent mechanisms (for example by AMPK phosphorylation and activation) (2) and increasing peripheral insulin sensitivity via HDL anti-inflammatory actions within the tissues themselves (for example, adipose tissue, muscle, liver) (3) and macrophages (4). The actions of HDL in metabolic tissues and macrophages might result from direct signalling events and via lipid removal through reverse cholesterol transport, with subsequent improvement of inflammation. From Drew et al, 2012.[306]

Indeed, infusions of rHDL particles reduced plasma glucose, increased insulin secretion and improves the homeostatic model assessment (HOMA) index compared with placebo.[307] Based on this study and on cell culture experiments (skeletal muscle cells), those authors suggested the infusions of rHDL increases skeletal muscle glucose uptake via activation of AMPK pathway, this effect involves the binding of ABCA1, maintenance of cholesterol homeostasis in pancreatic β-cells and stimulation of insulin secretion.[305,307]

The beneficial effect of HDL and ApoA-I on glucose metabolism and insulin resistant states are not limited to their actions on β-cells, skeletal muscle cells and monocyte but may be also target adipocyte. Thus, both over-expression of ApoA-I and treatment with ApoA-I mimetic peptide D-4F, reduces white fat mass, improve insulin resistance and diabetes in mice who develop obesity upon feeding on a high fat diet.[308] HDL-induced 5' AMP-activated protein kinase (AMPK) activation in adipose tissue may led to the inhibition of fatty acid lipolysis and oxidation as observed in patients with type 2 diabetes infused with rHDL.[309]

1.3.3 Functionally defective HDL

HDL particles progressively lose their normal biological functions and acquire altered properties as a result of modification in HDL composition, structure and metabolism that occur in the context of high cardiovascular risk.[310] Such altered HDL particles have been termed “dysfunctional HDL”,[311] and HDL has been proposed to possess “chameleon-like properties” (Figure 12).[216,312,313]

Compared with normal fully functional HDL, the composition of both the protein and lipid components of HDL can be modified in metabolic conditions associated with accelerated atherogenesis and enhanced cardiovascular risk. In such diseases states, HDLs become depleted in ApoA-I, CE, PON1 and LCAT and enriched in ApoE, ApoC-I, ApoC-III, free cholesterol, free fatty acids, lysophosphatidylcholine, isoprostanes, sphingolipids, TG, complement C3, C9 and SAA, during the acute phase, displaying covalent modifications as a result of oxidation and/or glycation (Figure 12).[310,314]

The diminution in HDL ApoA-I levels in inflammatory states is associated to both decreased hepatic ApoA-I synthesis and to its replacement in HDL particles by SAA, which may become the major HDL apolipoprotein during the acute phase.[312,314]

In addition, ApoA-I can undergo other modifications in the circulation, it aa residues like tyrosine, metionine, cysteine and lysine can be selectively modified under the action of pro-oxidants secreted by arterial cells.[315-318]

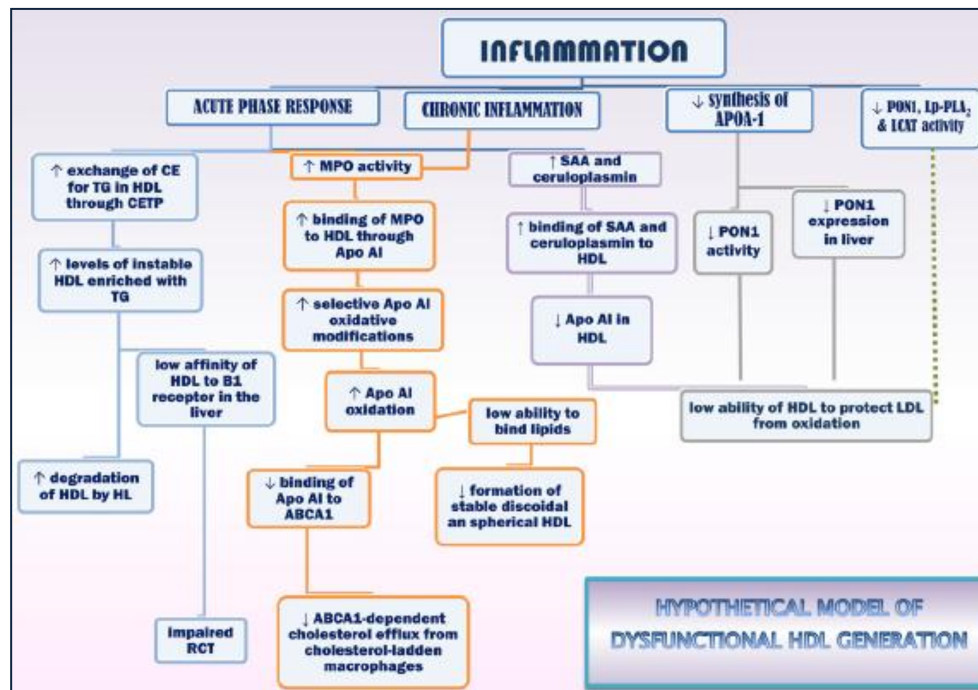


Figure 12. Proposed model for generating dysfunctional HDL. From Otocka-Kmiecik et al, 2012.[319]

MPO is an enzyme released during degranulation of neutrophils and monocytes, that uses hydrogen peroxide to generate chlorinating and nitrating oxidants which play an essential role in killing microorganisms. However, these reactive species can also modify host proteins and lipids. MPO is a major source of chlorinating and nitrating oxidants in the arterial wall and is enriched in human atheroma.[320] It has been shown to alter the function of HDL, in particular binds to ApoA-I *in vitro* and *in vivo* and produces similar patterns of oxidized aa residues.[315,320,321] In fact, the modified ApoA-I are unable to interact with the ABCA1 which also leads to impairment of other functions such as the ability to bind lipids.[315] Such modifications of ApoA-I display altered immune properties an can be quantitatively detected by specific antibodies.[322,323] Besides, ApoA-I oxidation may lead to its fragmentation, as well as oligomerization, as take place in aging.[324]

In addition to the oxidative modification, ApoA-I can be non-enzymatically glycated under conditions of chronic hyperglycemia, as in diabetic patients[325] or in aged patients.[326] Non-enzymatic glycation of proteins is a post-translational modification produced by a reaction between reducing sugars and amino groups located in lysine and arginine residues

or in the N-terminal position. Highly reactive glucose-derived dicarbonyl compounds, such as methylglyoxal, may account for the formation of glycated ApoA-I.[327] Glycation ApoA-I may result in severe structural changes associated with lower binding affinity to phospholipid, building discoid HDL with decreased α-helical content.[328] Furthermore, it may adversely affect RCT due loss of ApoA-I ability to activate LCAT,[183] and also the anti-inflammatory properties via a reduced capacity to inhibit NF-κB activation and ROS formation.[327,329]

HDL-associated enzymes, including PAFAH, PON1, and LCAT can become dysfunctional and/or depleted under inflammatory conditions,[227,330-332] in metabolic diseases involving low HDL levels (type 2 diabetes, metabolic syndrome).[333,334]

During the acute phase response there is a reduction of PON1 activity, probably as result of displacement and replacement of PON1 by SAA.[335] Moreover decreased PON1 activity may be caused by enzyme inactivation as a consequence of oxidation,[336-340] glycation[341,342] and /or homocysteinylation.[343,344]

The activity of both HDL-associated enzymes LCAT and PAF-AH may be also diminished under conditions such as dyslipidemia, insulin resistance, inflammation, infection and autoimmune disease.[345-349]

Even though apolipoproteins and enzymes are the most important determinants of altered HDL function, it may considerably be influenced by changes in HDL lipid composition. Enrichment in TG with depletion of CE in HDL core is the most frequent abnormality of HDL lipid composition.[314,346,350] Furthermore, the acute-phase HDL also contain high levels of nonesterified fatty acids, lysophosphatidylcholines and isoprostanes compared with normal HDL.[314] Also HDL lipids can also suffers modification and being oxidized *in vivo* with formation of biologically active compounds.

Even HDL formation itself may be also modified since it occurs a reduction of hepatic production of ApoA-I and defective ApoA-I lipidation upon interaction with ABCA1. Thus the defective formation of large HDL increases renal clearance of ApoA-I and subnormal HDL-C levels, and can also be caused by diminished cholesterol esterification by LCAT.

All of the previously described anti-atherogenic properties of HDL, such as cholesterol efflux capacity, anti-oxidant, anti-inflammatory, vasodilatory, antithrombotic and cytoprotective activity can become impaired under conditions favouring the accelerated atherogenesis and enhanced CVD risk.

The degree of loss of normal HDL function (i.e., defective function) compared with the absence of this function (i.e., dysfunction) depends on the assay used to characterize HDL functionality. In fact, HDL can be dysfunctional (complete loss of the capacity of HDL to perform normal anti-atherogenic function) in cell-based or cell-free assays aimed at measuring anti-inflammatory activity[311,351] and vasodilatory funtions.[352] In contrast, measurements of antioxidative activity[330-334] or cholesterol efflux capacity[346,353] demonstrate a diminished levels of the HDL normal anti-atherogenic functionality rather than a complete dysfunction.

1.3.4 Epidemiology of cardiovascular risk in relation to low HDL-C

The Framingham Heart Study conclusively demonstrated that low HDL-C is a risk factor for CAD independently of LDL-C (Figure 13): at any level of LDL-C, a decrease in HDL-C increases the risk of CAD . [354]

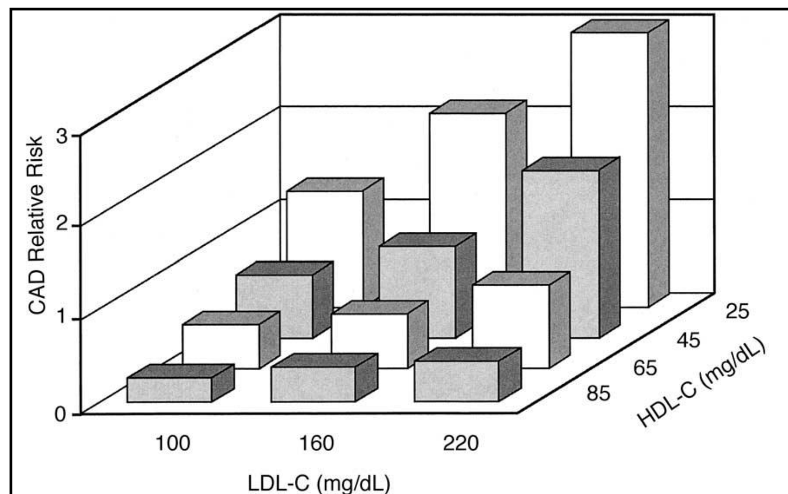


Figure 13. Coronary artery disease (CAD) risk predicted by high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in the Framingham Heart Study. Modified from Gordon et al, 1977.[354]

Subsequent studies of large populations provide even more evidence to support the role of low HDL-C as a CVD risk factor. Thus, simultaneous analysis of four American prospective studies (Framingham Heart Study, Lipid Research Clinics Prevalence Mortality Follow-Up Study, Coronary Primary Prevention Trial Placebo Group and Multiple Risk Factor Intervention Trial) has indicated that a 1 mg/dL increase in HDL-C was associated with a significant reduction in CAD risk of 2% in men and of 3% in women.[355]

Plasma HDL-C levels were also found to be an important predictor of event rates in clinical trials using statins.[356,357] Although the evidence for the utility of HDL-C as a risk marker in patients treated with lipid-altering therapy has been conflicting depending on the extent of covariate adjustments.

Large multivariable analyses using the Systematic Coronary Risk Evaluation (SCORE) dataset containing HDL-C levels of 104961 individuals (45% women) without pre-existing CHD from 7 pooled European prospective studies, confirmed the inverse, independent, strong and graded relationship between HDL-C and both CVD and CHD mortality.[358]

Of 58 prospective studies that providing multivariate assessments of the associations between low HDL-C and cardiovascular risk, 31 found a significant inverse association for all cardiovascular outcomes and subpopulations studied, whereas 17 found a significant association for some cardiovascular outcomes and/or subpopulations assessed.[359] Another large-scale epidemiologic study in which HDL-C was evaluated as a risk marker on 302430 individuals without initial vascular disease, from 68 long-term prospective studies, mostly in Europe and North America confirmed in multivariate models adjusted for both nonlipid and lipid (TG and non-HDL-C) risk factors that HDL-C was inversely associated with CHD events. For every 15 mg/dL increase in HDL-C concentration, the risk of a CHD event was reduced by 22% (95% CI, 18%–26%).[360]

These data leave no doubt regarding the role of low HDL-C as major cardiovascular risk factor. Thus, HDL-C has become an important component of algorithms to assess the global cardiovascular risk of patients such as the Framingham risk prediction tool, the PROCAM score and the SCORE approach.[361,362] Furthermore HDL is also a target for therapeutic intervention and for the definition of treatment goals.

1.3.5 Pharmacologic modulation of HDL-C

Lipid management guidelines are still focused on decreasing LDL-C levels using statins, which inhibit HMG-CoA reductase (a key enzyme in cholesterol biosynthesis), as the primary target for reducing CHD risk. However, despite the generalized use of statins aggressive LDL-C lowering resulted only in a reduction of 30% to 40% of the clinical events and high residual cardiovascular risk (approximately 60-70%) continue to persist (Figure 14).[78,363-365]

Therefore, to further reduce atherosclerotic cardiovascular events, increasing attention is now focused on HDL as a potential target for atherosclerosis management.

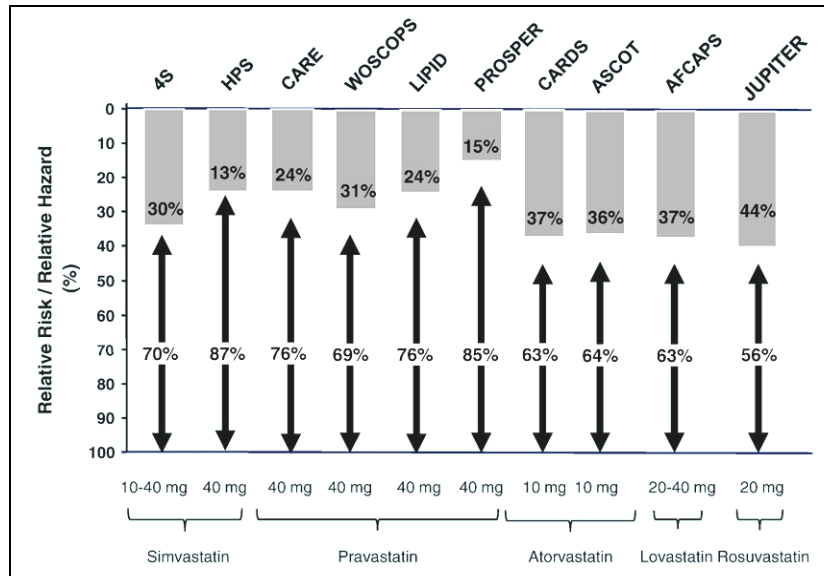


Figure 14. Residual cardiovascular risk despite statin treatment. 4S=Scandinavian Simvastatin Survival Study; HPS=Heart Protection Study; CARE=Cholesterol and Recurrent Events; WOSCOPS=West of Scotland Coronary Prevention Study; LIPID=Long-termIntervention with Pravastatin in Ischemic Disease; PROSPER=Prospective Study of Pravastatin in Elderly at Risk; CARDS=Collaborative Atorvastatin Diabetes Study; ASCOT=Anglo-Scandinavian Cardiac Outcomes Trial–Lipid-lowering Arm; AFCAPS=Air Force/Texas Coronary Atherosclerosis Prevention Study; JUPITER=Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin. Fom Chapman et al, 2010.[366]

1.3.5.1 Current available therapies for increasing HDL-C concentrations

The current pharmacologic options available for raising HDL-C levees include statins, fibrates and niacin.

1.3.5.1.1 Statins

Statins are competitive antagonist of HMG-CoA reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. Statins, therefore by competing directly with the endogenous substrate for the active site of the enzyme effectively reduce serum cholesterol levels. In addition they reduce serum cholesterol levels by upregulating hepatic receptor-mediated LDL-C clearance.

The major effect of statin is to effectively diminish plasma levels of ApoB-containing lipoproteins, principally LDL (20-60%) (Figure 15), but also IDL, VLDL and quilomicrons. The lowering of intracellular cholesterol levels leads to the activation of SREBP transcription factors, which activate the transcription of a number of genes involved in the clearance of LDL particles from plasma, such as the LDL receptor.[367] Statins also lower plasma TG due to a diminished VLDL production, probably as a consequence of less availability of cholesterol for

VLDL and quilomicrons assembly.[368,369] In addition, they may lower TG due to a strong induction of receptor-mediated remnant clearance.

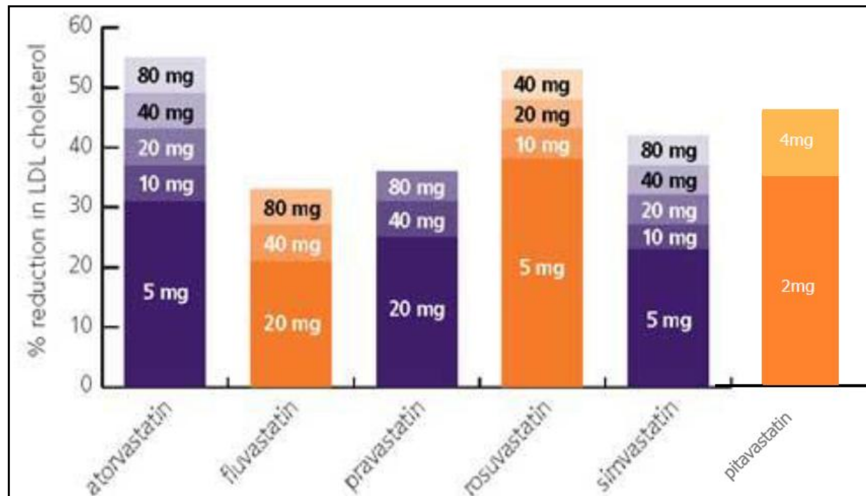


Figure 15. Percentage reductions in serum LDL cholesterol levels according to statin and daily dose. Summary estimates from 164 randomised placebo controlled trials. Adapted from Law et al, 2003.[370]

Statins only modestly raise HDL-C levels (by 3% to 15%) [199, 371-373]. Individual statin are not identical in their potency to increase HDL-C (Figure 16) with rosuvastatin an pitavastatin being the most potent compounds.[199]

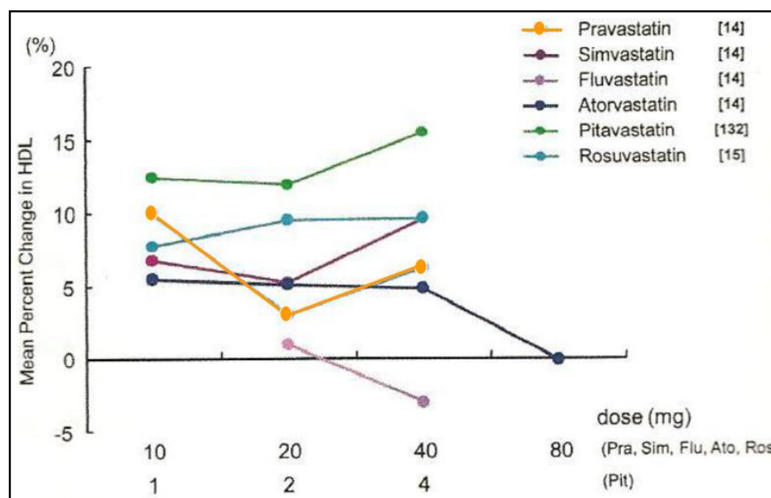


Figure 16. Effect of each statin on HDL-C levels. From Yamashita et al 2010.[199]

The differential effects of individual statins on HDL are further highlighted by the finding that increases in HDL-C is positively related to statin dose with rosuvastatin and simvastatin but inversely related to dose with atorvastatin, at least within the dose range of 20 to 80 mg/day.[373-375]

It has been difficult to clinically dissociate the benefits of the increased HDL-C from LDL-C lowering since the studies were designed to address LDL-C as the principal mechanism

of action of the statins. However the effects of statins on HDL-C levels may be attributed in part to inhibition of Rho-signalling pathways with activation of PPAR-α and consequent stimulation of ApoA-I production (Figure 17).[372,376] Statins also reduced circulating levels of CETP, and also importantly the rate of CETP-mediated CE transfer from HDL to VLDL secondary to reduction in the latter.[377,378] Statins, however, are not known to be direct inhibitors of CETP.

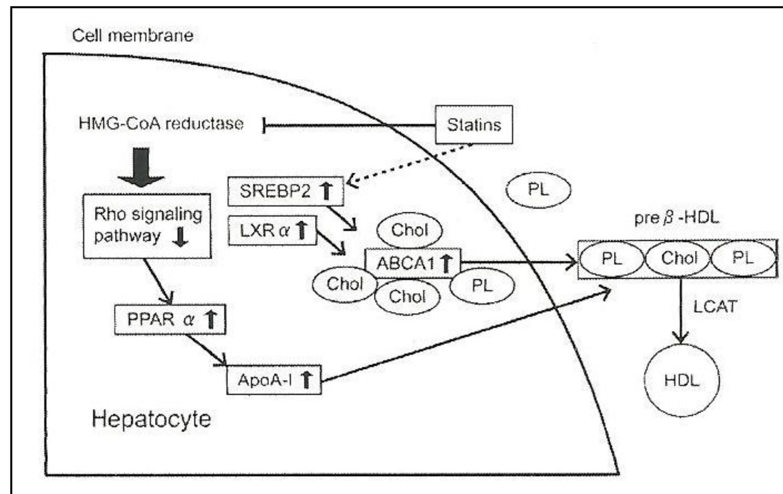


Figure 17. Mechanism of up-regulation of ApoA-I production by statins and effect on ABCA1. From Yamashita et al 2010.[199]

Importantly, statins exert multiple pleiotropic effects, as they inhibit the prenylation of proteins, such as Ras and Rho that activate the MAPK cascade or NF-κB pathway, that include anti-inflammatory, antiproliferative and antithrombotic effects (Figure 18) (For review see[379]).

Furthermore, statins seems to improve anti-oxidant actions of HDL by increasing the activity of HDL-associated enzymes as demonstrated for PON1. The increased PON1 activity was clinically confirmed with the administration of fluvastatin,[380] simvastatin,[381,382] atorvastatin[383,384] and rosuvastatin.[385] Statin effect on PON1 activity seems to be independent of HDL elevation.

In vitro studies showed that pitavastatin induces PON1 expression through the activation of the p44/42 MAPK signalling cascade in hepatoma cells, suggesting a beneficial effect on HDL funcionality.[386]

Previously , Deakin et al[382] observed that simvastatin was able to modulate *in vitro* the expression of PON1 regulated by SREBP-2 and increase serum PON1 concentration and activity in human hepatic HepG2 cells.

Statins are generally well tolerated; the most common adverse effects involve muscle problems such as myophathy and/or myositis with potential for rhabdomyolysis, raised liver enzymes (alanine transaminase ALT and aspartate transaminase AST), central nervous system effects, and the appearance of diabetes.[387]

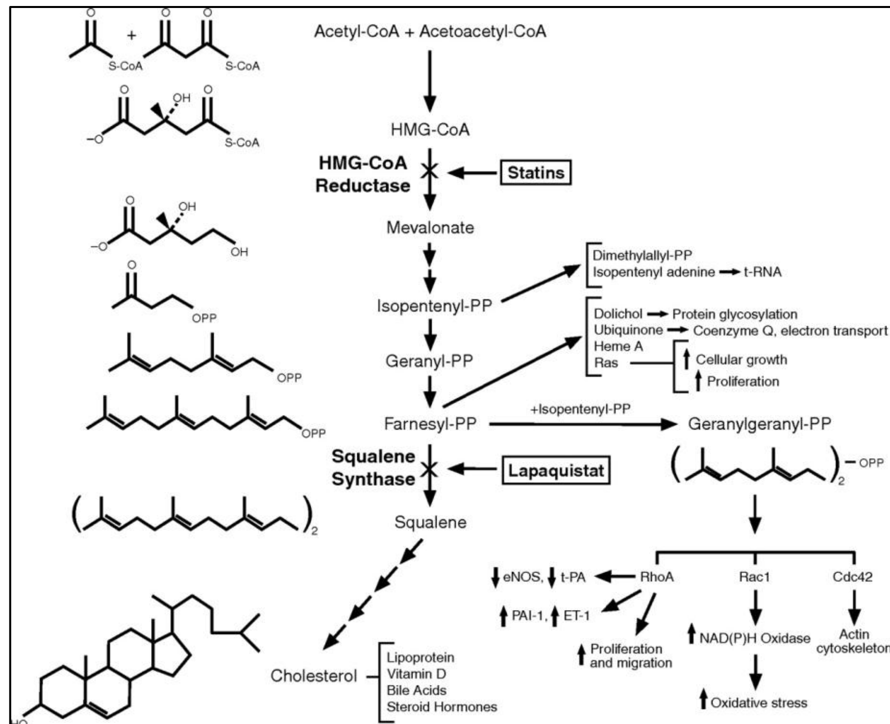


Figure 18. Cholesterol biosynthetic pathway. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by statins decreases the synthesis of isoprenoids and cholesterol. PP pyrophosphate; BMP-2: bone morphogenetic protein-2; eNOS: endothelial nitric oxide synthase; t-PA: tissuetype plasminogen activator; ET-1: endothelin-1; PAI-1: plasminogen activator inhibitor-1. From Liao et al, 2005.[379]

1.3.5.1.2 Fibrates

The fibrates are effective agents for managing dyslipidemia in particular elevated concentrations of TG-rich lipoproteins and low levels of HDL-C typically associated type 2 diabetes and the metabolic syndrome.[388]

Fibrates are PPARα agonists of moderate affinity that exert multiple effects on lipid and fatty acid metbolism.[389]

Fibrates bind to PPARα expressed in hepatocytes, endothelial and smooth muscle cells, monocytes, macrophages and the heart, and heterodimerizes with the RXR. The heterodimer PPARα/RXR binds to peroxisome proliferator response elements in the promoter regions of specific genes, activating the expression of these genes and thereby increasing protein expression (Figure 19).[390] Activation of PPARα by fibrates mediates

several effects such as the modulation of lipoprotein metabolism, coagulation, haemostasis and attenuation of inflammation.[391]

The primary effect of fibrates on plasma lipoprotein lipids involves a 30–50% reduction in TG levels and in general increase levels of HDL-C by 5–15%, depending on lipid phenotype and baseline concentration.[388,392] Fibrates may also reduce LDL-C potentially by up to 15-20% although the effect is variable, depending on the fibrate in question and on baseline lipid profile.[388,393]

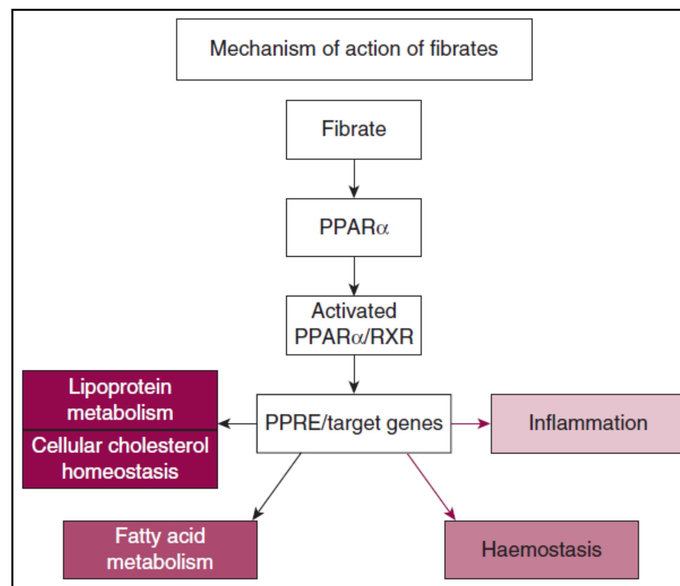


Figure 19. Mechanism of action of fibrates. From Chapmam 2006.[391]

Fibrates decrease circulating TG levels by increasing catabolism of VLDL (and chylomicrons) via induction of LPL and ApoA-V expression, and simultaneously, LPL action is potentiated due to attenuated hepatic ApoC-III expression, a well-known inhibitor of LPL. In addition they stimulate cellular fatty acid uptake and transformation to acyl-CoA derivatives, and by raising peroxisomal and mitochondrial β-oxidation, thereby leading to attenuated synthesis of fatty acids and TGs that results in a decrease in VLDL production.[390]

Fibrates increase HDL independently of TG reduction by direct up-regulation of the transcription of APOA1 and APOA2 genes, leading to increased synthesis of ApoA-I and ApoA-II, thereby enhancing the formation of new HDL particles.[394]

Another main aspect of the action of fibrates on HDL metabolism involves enhanced expression of ABCA1, a major regulator of HDL biogenesis in hepatocytes, and known to be up-regulated by the transcription factor liver X receptor (LXR), especially LXRα, which expression is further enhanced by activation of the PPARs.[395]

Studies in primary hepatocytes from mice have shown a down-regulation of hepatic SR-BI protein levels on treatment with fibrates.[396] Down-regulation of this receptor in the liver leads to decreased HDL clearance and would provide another mechanism by which fibrates could increase plasma HDL levels.

Interestingly the indirect inhibitory action of PPARα agonists on plasma CETP activity as resulted of reduced TG levels, can be, partly counteracted by enhanced CETP expression, as showed by effect of fenofibrate in increasing CETP mRNA, protein and activity in human CETP transgenic animal model.[397] Consistent with this result, fenofibrate increases circulating CETP levels in dyslipidemic humans.[398]

In addition, fibrates increased PON1 concentration and activity[399] and are involved in the redistribution of PAF-AH from ApoB containing lipoproteins to HDL in dyslipidemia patients, thereby lowering the pro-inflammatory potential of the enzyme.[400,401]

Despite such significant anti-atherogenic actions, clinical trials of cardiovascular risk reduction by fibrates have provide mixed results, some revealed beneficial effects (Helsinki Heart Study, VA-HIT and WHO trial),[402] whereas in contrast others (BIP and FIELD trial) are controversial.[402,403]

Disappointingly, HDL-C increase by fibrates is markedly attenuated by type 2 diabetes and typically does not exceed 5% .[366,403] The relatively weak HDL-C-raising effect of fibrates in this group of patients may, in part, reflect the increase of atherogenic Hcy plasma levees and thus down-regulating hepatic synthesis of ApoA-I.[404] In the FIELD trial PPARα agonists thus increase plasma Hcy levels and this were negatively correlated with increases in HDL-C and ApoA-I, but positively correlated with elevation in ApoA-II levels.[403,404] In fact, PPARα agonists that have more robust effect on HDL-C and ApoA-I and do not raise Hcy would therefore be desirable.

PPARα agonists can also attenuate the production of pro-inflammatory stimuli such as TNFα, interferon gamma (IFN-γ), IL-6 and IL-1, as well as the acute phase proteins, including fibrinogen and CRP.[405] PPARα activation decrease the expression of adhesion molecules to the endothelium, due to the reduction cytokine-induced VCAM-1 expression, leading to decreased leukocyte recruitment into the arterial wall.[406] Furthermore PPARα activators inhibit thrombin-induced endothelin-1 production and consequently decreases the proliferation of smooth muscle cells.[407]

In general, fibrate therapy is well tolerated, but may induce hyperhomocysteinemia with concomitant increase of the creatinine levels, a marker of renal dysfunction.[408,409] Association of some fibrates with statin may produce a increase in the risk of myopathy and rhabdomyolysis, due to interferes with statin glucuronidation that could lead to elevation in statin plasma concentrations.[410-412]

1.3.5.1.3 Niacin

Niacin (nicotinic acid), vitamin of the complex B3, has been used as a pharmacologic agent to regulate abnormalities in plasma lipid and lipoprotein metabolism since 1955.[413] Niacin is presently the most effective commercial available lipid-regulating agent to increase HDL-C levels. The HDL-C raising effect of niacin may reach to 35% of increase at a 2g/day dose, as well as substantially lowering TGs (20-40%), LDL-C (15-30%) and lipoprotein(a) (Lp(a)) (15-35%).[414-416]

The primary action of niacin is to transiently suppress the TG lipolysis in adipose tissue.[417,418] Nicotinic acid has been identified as a ligand for the human G-protein-coupled receptors GPR109A and GPR109B that signal through Gi-mediated inhibition of adenylyl cyclase (AC), resulting in a decrease in intracellular cyclic adenosine monophosphate (cAMP) levels.[419] This cyclic nucleotide is the principal mediator of adipocyte lipolysis (Figure 20) Lipolysis is increased when cAMP levels are elevated due to increased AC activity, for example, by β-adrenergic receptor activation or by decreased phosphodiesterase-mediated cAMP degradation.[420] Thus, the nicotinic acid-induced, GPR109A-mediated AC inhibition suppressing the prolipolytic effects of elevated intracellular cAMP levels and stimulation of protein kinase A (PKA), which phosphorylates a number of proteins, most notably hormone-sensitive lipase (HSL), which are required for TG hydrolysis.[416]

Another important lipase in this process is the adipose triacylglycerol lipase (ATGL), which hydrolyzes the TGs into FFA and glycerol. Thus, the antilipolytic action of nicotinic acid is likely mediated by activation of the Gi-coupled nicotinic acid receptor, impeding the cAMP/PKA signalling cascade thereby decreasing lipolysis and the subsequent release of FFA into the circulation. The flux of FFA to the liver constitutes the main substrate for hepatic TG synthesis, such TG may be either integrated into nascent VLDL particles and secreted into the circulation, or may alternatively be stored in the form of intracellular lipid droplets in the hepatocyte. Furthermore niacin inhibit the diacylglycerol acyltransferase 2, a key enzyme of

TG synthesis in human hepatocytes.[421] The inhibition of TG synthesis by niacin potentially results in the accelerated intracellular hepatic degradation of ApoB and subsequent decreased secretion of VLDL/LDL particles.

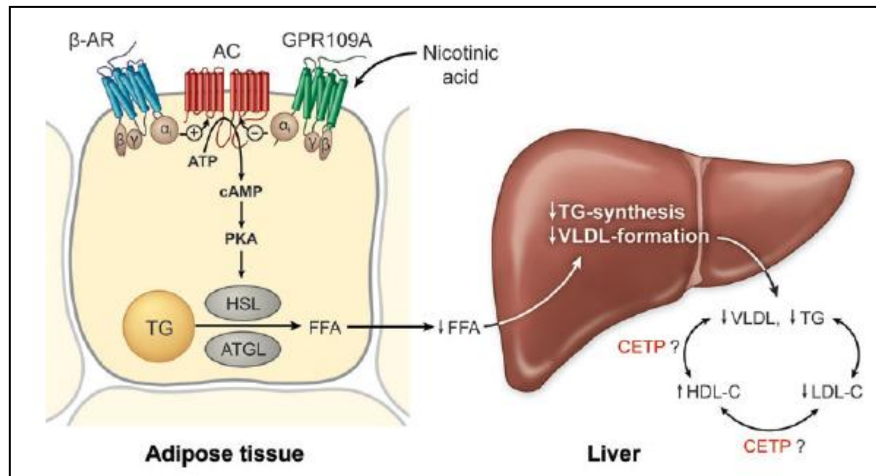


Figure 20. Mechanisms of nicotinic acid-induced changes in lipolysis of triacylglycerol (TG) on adipocytes and reduction hepatic very low density lipoprotein (VLDL) production. ATGL, adipocyte-triacylglycerol-lipase; CETP, cholesterol ester transfer protein; FFA, free fatty acid; HSL, hormone-sensitive lipase; PKA, protein kinase A; TG, triacylglycerol; AC, adenylyl cyclase; β-AR, β-adrenergic receptor. From Gille et al, 2008.[422]

The mechanism of the nicotinic acid–mediated increase in HDL-C levels is less clear. Most likely, the decrease in TG levels in ApoB-containing lipoproteins (LDL/VLDL) results in a decreased exchange between CE carried by HDL particles and TG in VLDL and LDL particles via CETP, resulting in an increase in HDL-C plasma concentrations. In parallel niacin may also promotes hepatic ApoA-I production and slows hepatic clearance of ApoA-I.[422,424]

Furthermore, another potential mechanism of niacin raising HDL-C involves the induction of cholesterol efflux via ABCA1 and ABCG1 from peripheral cells to HDL acceptors. In fact, niacin enhances transcription of ABC transporters via nuclear PPARγ-dependent pathway[425] and may promote cholesterol efflux from adipocytes to ApoA-I via ABCA1 mediated by LXR-α dependent mechanism.[426]

There is emerging interest in several potentially beneficial non-lipid mediated effects of niacin e.g. anti-inflammatory, anti-oxidant, antithrombotic and cytoprotective effects.

Niacin administration has been shown to inhibit TNF-α induced inflammatory response in cultured human endothelial cell by decreasing NF-κB activation, VCAM-1 and ICAM-1 expression and MCP-1 secretion.[427-429]

Furthermore, niacin also decreases CRP levels,[430] improves endothelial dysfunction,[431,432] improves the endothelial and leukocyte redox state *in vitro*, which

protected against ROS-induced endothelial dysfunction,[427,433] enhances plaque stability and reduces thrombosis.[434]

Niacin increases adiponectin, an adipokine with insulin sensitizing, anti-atherogenic, and anti-inflammatory properties.[435-437]

Recently, it has been reported that niacin inhibits vascular inflammation via mechanisms independent of its lipid-modifying effects.[433,437] Therefore in addition to its lipid modulation role these athero-protective qualities seem contribute to the improved cardiovascular outcomes demonstrated in patients treated with niacin.

However, the therapeutic potential of niacin has been limited by its adverse effects; cutaneous flushing phenomenon that occurs in 70–80% of the patients.

Niacin has also been criticized for dysregulation of glycaemia control,[438,439] elevation of liver enzymes[440] and uric acid level[441].

1.3.5.2 Emerging therapies for reducing CVD risk via HDL

The magnitude of the HDL-C elevations achieved by either lifestyle or any of the currently available pharmacologic options is small relative to the LDL-C reductions achieved by statins and the high residual cardiovascular risk (approximately 60-70%) continue to persist. Therefore, development and testing of new therapies that exploit the vascular protective effects of HDL constitutes a rational and complementary approach.

In recent years, a number of promising HDL-based therapies have emerged from intensive research (Figure 21).

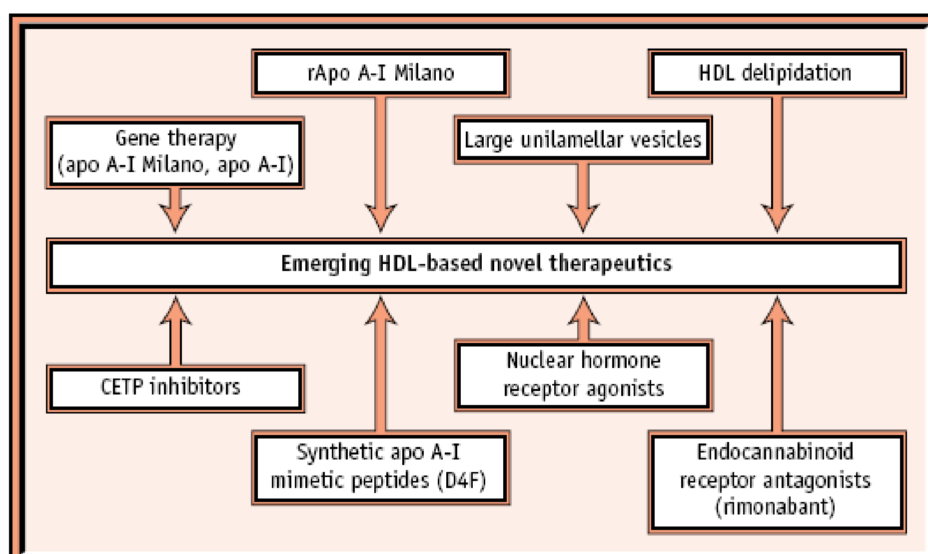


Figure 21. Emerging HDL-based therapies. From Shah 2007.[442]

This has led to several new drugs that are currently in various stages of development and testing (Phase I–III clinical trials). It is hoped that one or more of these new HDL-based therapies, if proven effective and safe, will become a part of the therapeutic option for management of dyslipidemia and against cardiovascular disease. These therapeutic approaches to raise HDL-C levels can target one or more of several mechanisms involved in HDL metabolism, including the production of ApoA-I or modification of intravascular remodelling of HDL particles.

For the purpose of this work I have included only the ones that consist in the administration of HDL components as they may have a direct association with the production of antibodies towards HDL complex.

1.3.5.2.1 Apo A-I/Reconstituted HDL (rHDL) Infusions

Despite intensive research, small molecules that could increasing endogenous ApoA-I expression without the multiple side effects of the above-mentioned substances remain elusive, an alternate strategy has been to directly infuse ApoA-I or rHDL into the circulation that might act as cholesterol acceptors.

ApoA-I Milano has been known for years to be a variant of the ApoA-I protein backbone of HDL particles that enhances the ability of to transport cholesterol out of the body. People from a small village in Italy who have this genetic variant have lower CHD events and longer. Recombinant form of ApoA-I Milano in a phospholipid complex (ETC-216) is being developed as an infusion product.

Infusions of wild-type ApoA-I/HDL and ApoA-I Milano were tested in animal models and were associated with attenuation, and even regression, of atherosclerosis in rabbit models.[443-446]

This effect has also been demonstrated in humans as shown in Table 4 which summarizes the clinical evidence of infusion of Apo-AI Milano and wild-type Apo-AI remarkable decrease of atheromatous burden.[239,447-449]

Besides this effect on acute plaque regression the ApoA-I/HDL infusions seems to exerts anti-inflammatory effects.[239,446]

Bringing this compound to clinical practice will require further large trials that evaluate its impact on cardiovascular events. However, due to its complexity the protein is difficult

and very expensive to produce and its use via the intravenous route limits its trial application compared to oral medication.

Table 4. Clinical evidence for ApoA-I/ HDL infusion. Adapted fom Redondo et al, 2011.[450]

Study	Patients	Administration	Results
Nissen et al, 2003[447]	123 post-acute coronary events (randomized)	Intravenous ApoA-I Milano	4.2% decrease of atheromatous plaque volume (measured by ultrasound)
Nicholls et al, 2006[448]	47 post-acute coronary events (randomized)	Intravenous ApoA-I Milano	4.6% decrease of internal elastic lamin (measured by ultrasound)
Tardif et al, 2007[449]	183 patients on coronariography (randomized)	Intravenous reconstituted wild-type HDL	3.4% decrease of plaque volume (measured by ultrasound) at follow up (not difference at baseline)
Shaw et al, 2008[239]	20 patients with claudication who underwent femoral endarterectomy (randomized)	Intravenous reconstituted wild-type HDL	Decreased expression of vascular cell adhesion molecule-1 and decreased lipid content

1.3.5.2.2 Synthetic Apo A-I mimetic peptides

ApoA-I mimetic peptides are short synthetic amphipathic peptides of 18–22 aa, which mimic the lipid-binding domain of ApoA-I and retain functional properties of ApoA-I, such as the ability to form complexes with lipids, promote cell cholesterol efflux, and activate LCAT.[451,452] They have the advantage of being relatively easy and cheap to synthesize compared with the full-length ApoA-I

The most promising ApoA-I mimetic peptide seem to be D4F, an orally active peptide with D-amino acids (which are resistant to gastric hydrolysis unlike L-amino acids) and 4 phenylalanine substitutions, has been shown to enhance the anti-oxidant and anti-inflammatory function of HDL and improve its cholesterol efflux ability without increasing plasma HDL-C levels in mouse models.[453]

The D4F peptide is also the only apolipoprotein mimetic peptide reported so far to undergo testing in human subjects. In a Phase I study of 50 patients with coronary heart disease received a single a single dose of 30, 100, 300, or 500 mg of unformulated D-4F. Even though no apparent toxicity was observed from this trial, there has been apprehension about whether long term use of D4F.[454]

Therefore the early optimism regarding the therapeutic potential of these mimetic peptides has been attenuated by the inability of any of these peptides to move to an advanced stage of clinical development.

An alternative therapeutic option may be to turn on endogenous ApoA-I synthesis. Such an approach would generate nascent HDL particles, which should carry out normal physiological functions. Nevertheless, the capacity to identify agents that selectively increase ApoA-I synthesis has proven to be a major challenge.

RVX-208 is a first-in-class, orally active, small-molecule that acts via an epigenetic mechanism leading to enhanced activity of the APOAI gene resulting in upregulation hepatic expression of ApoA-I and to increase systemic cholesterol efflux capacity in *in vitro* and *in vivo* models and in phase I/II clinical trials.[455-457]

Currently there is great expectation for the results of two Phase IIb trials with this ApoA-I stimulator the SUSTAIN (the Study of Quantitative Serial Trends in Lipids with Apolipoprotein A-I Stimulation) study that aims to evaluate the lipid efficacy, safety and tolerability of RVX-208, and the ASSURE (the ApoA-I Synthesis Stimulation and Intravascular Ultrasound for Coronary Atheroma Regression Evaluation) study that aims to evaluate the effect of RVX-208 on plaque burden.[458]

1.4 Immunity in Atherogenesis

1.4.1 Overview

Once considered a feature of the long and relentless aging process, atherosclerosis is regarded now as a chronic inflammatory and auto-immune disease in which all branches of the immune system are involved at each stage of the atherosclerotic process. [54-56]

Some of the most remarkable data in support of a link between immune response and atherosclerosis came from epidemiologic studies of patients with auto-immune diseases. Patients with rheumatoid arthritis (RA) have a 2- to 5-fold increase in cardiovascular morbidity and mortality,[459] and patients with SLE have a 9 to 50-fold risk of developing atherosclerotic CHD than the normal population.[460] Interestingly, patients with SLE, RA and other inflammatory syndromes share several auto-immune phenomena with atherosclerotic patients, such as immune dysfunction, inflammation and endothelial activation as a consequence of chronic therapies.[461-463]

The study of immunity in atherogenesis was boosted with the creation of animal models of atherosclerosis. Hypercholesterolaemic mice deficient in specific components of the innate and adaptive immunity show that the net effect of both systems is pro-atherogenic.[464-466] These findings may explain why atherosclerosis has such a major impact in systemic auto-immune diseases as they are characterized by a dysregulated and hyperactive immune system. However, immune activation and hyperesponse can still be a major player in atherogenesis event outside the context of auto-immune diseases.

Therefore, these particular diseases might constitute a logical model for the study of the complex mechanisms of atherogenesis.

1.4.2 General innate immune response

The innate immune system is the first line of defence against bacteria, viruses, and substances that appear foreign and harmful. Innate immunity is mediated by leucocytes, mast cells, natural killer (NK) cells and antigen presenting cells (APCs), such as macrophages and DCs.

Inflammation in physiological conditions is a self-limiting ancient protective mechanism that defends the host from invading pathogens. However it has become apparent that the innate arm of the immune inflammatory response does not involve only specific responses and phagocytosis. It relies on a set of pattern-recognition receptors (PRRs) that include

scavenger receptors (SRs) and TLRs that are able to sense highly conserved pathogen associated molecular patterns (PAMPs) and endogenous products of tissue injury and inflammation, with a greater degree of specificity than previously believed and has the ability to discriminate self versus foreign pathogens.[467,468]

TLRs expression is not constitutive and may be regulated by multiple factors including cell differentiation and the presence of their cognate ligands. Once activated, the TLR recruits adaptor proteins, being the myeloid differentiation factor 88 (MyD88) common to all TLR signalling pathways (with the exception of TLR3), that triggers the activation of pro-inflammatory transcription factors (NF-kB, AP1, CREB, c/EBP, and IRF), resulting in stimulation of the expression of various inflammatory molecules, such as IL-6, TNF-α, and IL-1β, chemokines, proteases, free oxygen radicals, ecosanoids (leukotriene B4, LTB_4) and costimulatory molecules.

In the circulation, these pro-inflammatory mediators induced by SRs and TLRs stimulation may subsequently activate hepatic (low grade) acute phase responses. For instance, systemic IL-6 can induce expression of the acute phase proteins such as CRP and SAA, whose elevated levels are associated with increased cardiovascular risk.[469]

Furthermore, recent evidence indicates that activation of the innate immunity is necessary to stimulate complete maturation of DCs and is essential to the induction of adaptive immune responses.

TLR-induced DCs maturation is characterized functionally by increased potency of DCs as APCs which results from the upregulation of major histocompatibility complex (MHC) class II and costimulatory molecules (CD40, CD80, CD86), as well as by cytokine production which leads to an efficient T cell activation (Figure 22).[470,471]

1.4.3 General adaptative immune response

The adaptive immune response, evolved later than the innate immune response, is composed of specialized antigen-specific lymphocytes T and B cells that require presentation of antigens, costimulators and particular cytokines from innate immune cells to become activated. Adaptive responses occur following recognition of an antigen by T-cell receptors (TCRs) on T cells and B cell antigen receptor (BCRs) on B cells. Once the adaptive immune cells have been activated, memory cells will be generated to rapidly respond in case of a new infection by the same pathogen.[472,473]

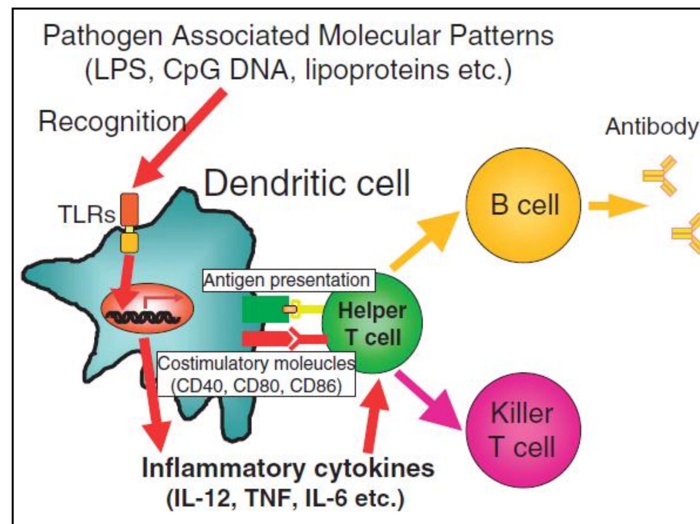


Figure 22. Interaction between innate and adaptative immunity. Dendritic cell (DCs) maturation and consequent T cell activation. Activation of toll-like receptors (TLRs) by recognition of pathogen associated molecular patterns (PAMPs) in DCs results in the stimulation of the expression of various inflammatory cytokine and costimulatory molecules and upregulation of major histocompatibility complex (MHC) class II, which are all necessary for the DCs antigen presentation and efficient T cell activation. From Akira, 2009.[471]

Adaptive immune responses are carried out by lymphocytes that mediate two types of response: a cellular immunity (cell-mediated immune responses) and a humoral immunity (antibody responses) that are carried out by T cells and B cells, respectively.

T cells can be divided into subsets based on their cell surface antigens, i.e. into $CD4^+$ T cells, also called T helper (Th) cells, regulatory T cells and cytotoxic $CD8^+$ T cells. Both $CD4^+$ and $CD8^+$ T cells are activated by mature antigen presenting DCs, however $CD8^+$ T cells are activated by peptide-antigens presented on MHC class I molecules and also requires costimulation via CD28 (on the T cell) and CD80 or CD86 (on the APC) and secretion of cytokines from $CD4^+$ T cells.[474]

$CD4^+$ T cells are key players in orchestrating immune responses by differentiation into specific Th subtypes with distinct and adequate immune responses. In addition, Th cells participate in humoral immune responses, by “helping” B cell isotype switch, resulting in the generation of antigen specific antibody production.

Once an antigen is recognized by the BCRs on B cells it transmits signals directly to the cell's interior and triggers a receptor-mediated endocytosis which allows the antigen to be processed and displayed as peptide fragments bound to MHC class II molecules at the B cell surface. B cell can then be recognized by antigen-specific armed Th cells, leading to the expression of the B cell stimulatory molecule CD40 ligand (CD40L) on the Th cell surface and to the secretion of the B cell stimulatory cytokines IL-4, IL-5, and IL-6, which drive the

proliferation and differentiation of the B cell into antibody-secreting plasma cells (plasma B cells) and memory B cells (Figure 23).[475]

In addition to producing antibodies, B cells play an important role in T cell responses via antigen presentation[476] and cytokine production.[477]

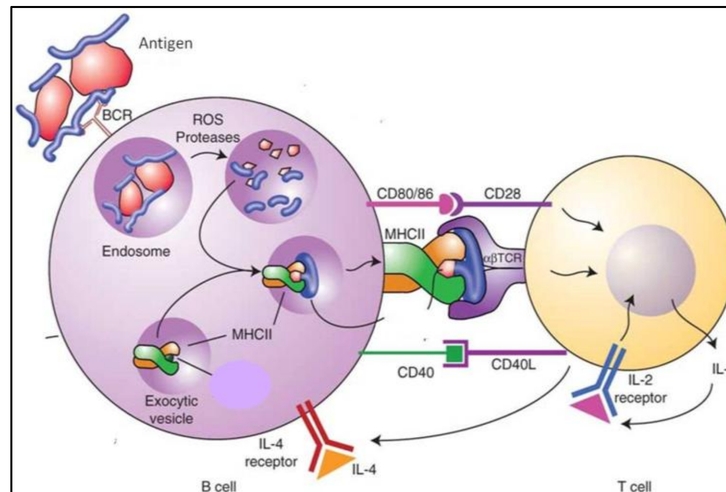


Figure 23. T cell-dependent B cell activation. Once a B cell encounters its cognate antigen, the B cell receptor (BCR) delivers the antigen to intracellular sites where it is degraded and returned to the B-cell surface as peptides bound to MHC class II molecules. The peptide:MHC class II complex can be recognized by antigen-specific armed helper T (Th) cells, leading them to make B cell stimulatory molecule CD40 ligand (CD40L) and cytokines such as IL-4 that cause the B cell to proliferate and differentiate into antibody-secreting plasma cells (plasma B cells) and memory B cells. Adapted from Avci et al, 2011.[478]

1.4.4 The immune response in atherogenesis

1.4.4.1 Innate immune response in atherogenesis

Activation of the innate immune mechanisms are implicated early in atherogenesis and in the inflammatory response.[479] The most abundant cell types within the atherosclerotic plaque are innate immune cells, such monocytes-macrophages, DCs and endothelial cells that can express a large repertoire of SRs classes and TLRs types.[467,480] These recognize and internalize a broad range of molecules and particles such as bacterial endotoxins, stress proteins, apoptotic cell fragments, DNA motifs, modified lipoprotein as oxLDL, HSP60 (Table **5**).[481] Although other antigens may also be important, recent data suggests that major atherosclerosis-relevant antigens consist of neoepitopes generated as a consequence of oxidative reactions, as occur when oxLDL is formed or when cells undergo apoptosis.[482]

Table 5. Several ligands for the different pattern-recognition receptors (PRRs), scavenger receptors (SRs) and toll-like receptors (TLRs). From Hansson et al, 2002.[54]

Ligand	Scavenger Receptor (SR)	Toll-Like Receptor (TLR)
LPS	SR-A	TLR2, TLR4
Lipoteichoic acid	SR-A	TLR2, TLR4
Acetyl-LDL	SR-A, MARCO, SR-EC	?
Oxidized LDL	SR-A, CD36, SR-PSOX, LOX-1	?
HSP60	?	TLR2, TLR4
Bacterial CpG DNA	?	TLR9

Intracellular cholesterol that accumulates after SRs –mediated uptake of modified LDL might activate cytoplasmic caspase-1-activating protein complexes (inflammasomes), being the most studied NLRP3, that promote maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Figure 24).[483]

TLRs also recognise modified LDL triggering the intracellular signalling cascade described previously that culminate with the expression of several pro-inflammatory molecules such as cytokines, chemokines, eicosanoids, proteinases, oxidases and costimulatory molecules (Figure 24).

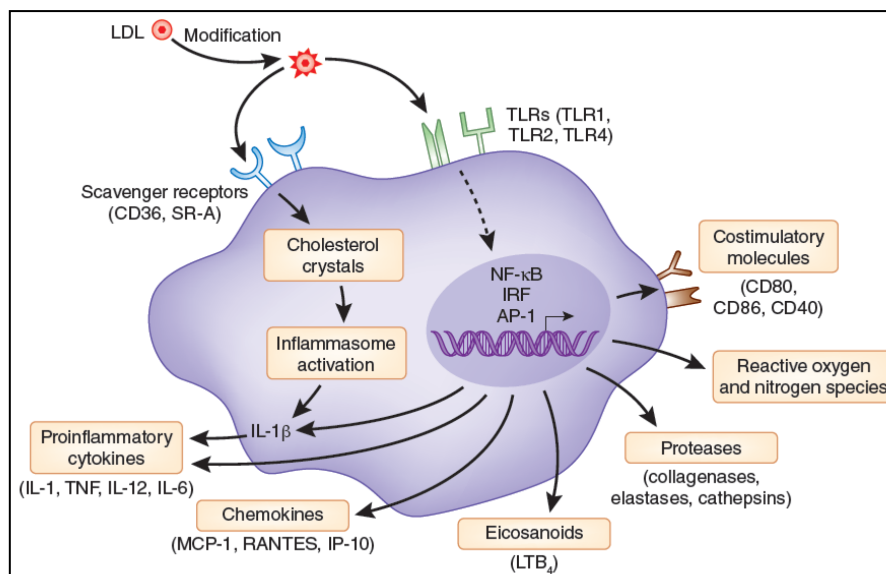


Figure 24. Activation of innate immune response in atherosclerotic lesion Uptake of modified LDL particles such as oxLDL through scavenger receptors (SRs) leads to the intracellular increase of cholesterol that can trigger the inflammasome, inducing the secretion of IL-1β. Toll-like receptors (TLRs) also recognise components of modified LDL, triggering an intracellular signaling cascade that leads to the expression of a series of genes encoding pro-inflammatory molecules, including cytokines, chemokines, eicosanoids, proteinases, oxidases and costimulatory molecules. From Hansson and Hermansson, 2011.[484]

In addition to stimulate complete maturation of DCs, activation of monocytes-macrophages are also a crucial link between the innate and adaptive arms of the immune response either on presenting foreign antigen to T cells as by subsequently responding to cytokines produced by activated T cells, promoting inflammatory responses, stimulation of lesion progression, and increase the risk of plaque rupture.[485]

1.4.4.2 Adaptative immune response in atherogenesis

1.4.4.2.1 Cellular immunity

Several studies have shown an accumulation of activated T lymphocytes in atherosclerotic plaques strongly suggesting that they influence disease progression.[486-488]

APCs within the atherosclerotic plaque trigger a predominantly Th1 cell response characterized by secretion of IFN-γ[489-491] which activates monocytes-macrophages, DCs, improves the efficiency of antigen production and promotes further Th1 polarizatin.[484] Furthermore, IFN-γ induces the expression of the SRs in macrophages increasing the oxLDL uptake, and inhibits the ABC transporters leading a defective cholesterol efflux, thus contributing to the formation of foam cells.[492] In addition to IFN-γ activated monocytes-macrophages and DCs produce others inflammatory cytokines such as IL-1, IL-12, IL-15, IL-18, and TNF-α and release proteases that reduce the stability of plaque by degrading the extracellular matrix and induce migration of SMCs from the media. These cells may also produce prothrombotic and procoagulant factors that directly precipitate the formation of thrombus at the site of plaque rupture (Figure 25 and Figure 26).

The role of Th2 cells in atherogenesis is poorly understood and data are inconsistent depending on the stage and ⁄ or site of the lesion, as well as on the experimental model (Figure 26). The Th2 cytokine IL-4 has been reported to be pro-atherogenic[493] or have no effect.[494] On the other hand, IL-13 was reported to be atheroprotective,[495]as was IL-5.[496]

The third distinct lineage of Th cells are the Th17, characterized by the production of the inflammatory cytokine IL-17,[497] and several other pro-inflammatory cytokines (IL-22 and IL-23) and are differentiated from activated naive T cells in the presence of TGF-β and pro-inflammatory cytokines, such as IL-6.

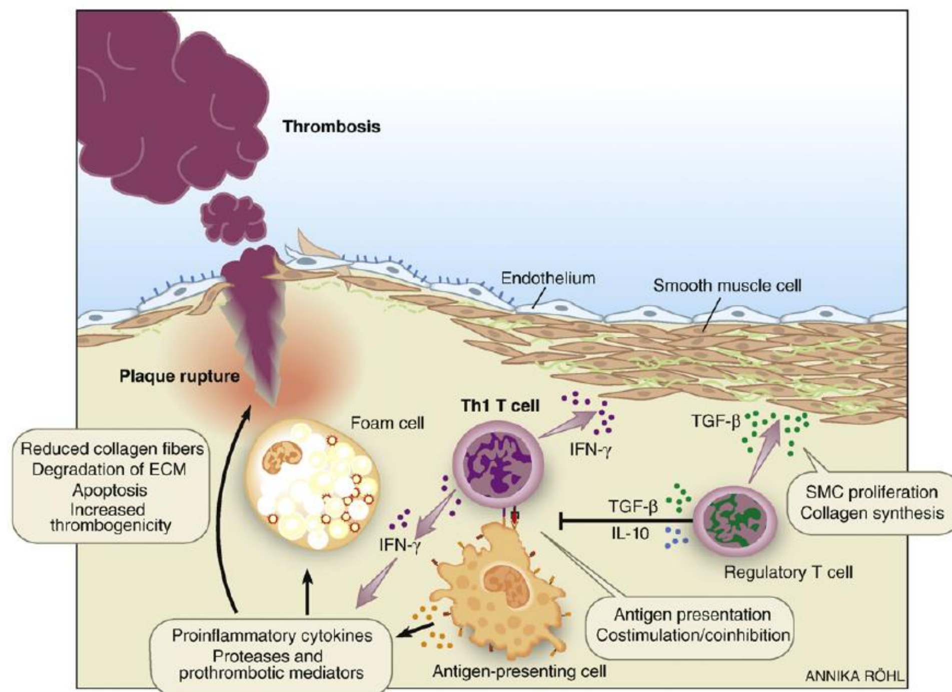


Figure 25. Activation of T cells in atherosclerotic lesions. It is characterized by a type 1 helper T (Th1) effector cells response that secrete interferon-gamma (IFN-γ) which improves the efficiency of antigen presentation and stimulates synthesis of inflammatory cytokines. Various cytokines and mediators produced by macrophages and T lymphocytes reduce the stability of plaque and induce migration of SMCs from the media and their proliferation and extracellular matrix production. They may also increase prothrombotic and procoagulant factors that directly accelerate the formation of thrombus at the site of plaque rupture. From Andersson et al, 2010.[473]

Th17 cells are involved in the promotion of inflammatory auto-immune diseases.[498] Nevertheless the effects of Th17 cells or IL-17 in human atherosclerosis are inconsistent, likely reflecting poorly understood relationships between Th1 and Th17.[499] Although accumulation of Th17 cells and IL-17 has been observed in murine[500,501]and human[502,503] atherosclerotic lesions, conflicting results with pro-[503,504] and anti-atherogenic (Figure 26).[505,506]

The discrepancies in the data may be relate to the existence of IL-17–producing T cells that also produce IFN-γ,[502,507] or to plasticity of Th17 cells, which can redifferentiate into Th1 cells.[508,509]

While overall T effector cells aggravate atherosclerosis, certain subsets of T cells within certain contexts can limit inflammation and counteract plaque complication. Regulatory T cells (Tregs), which actively maintain immunological tolerance to self and nonself antigens, are powerful inhibitors of atherogenic process by secreting anti-inflammatory cytokines such as IL-10, TGF-β, and IL-35 (Figure 25 and Figure 26).[510]

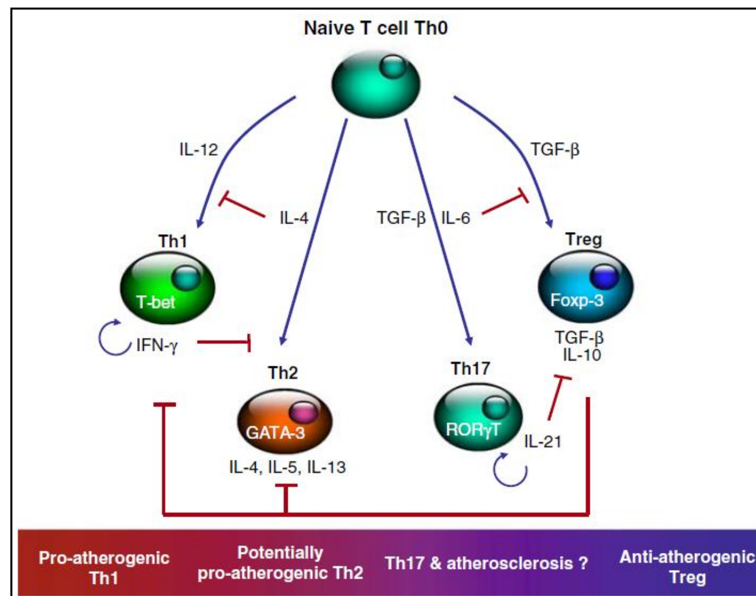


Figure 26. Naive T-cell differentiation into specific T helper (Th) substes involved in atherosclerosis and its complex interactions. From Ait-Oufella et al, 2009.[511]

The atheroprotective role for Tregs in animal models of atherosclerosis has been supported by studies in which Tregs depletion,[511,513] ablation[514] or genetic inactivation/blockade of Tregs, principal effector cytokines IL-10 and TGF-β[510,515] significantly, aggravates atherosclerosis. On the other hand injection or induction of the Tregs leads to diminished disease.[511,516,517]

Recently, the importance of Tregs in atherosclerosis development was further showed by the Tregs-mediated suppression on monocytes-macrophages foam-cell formation though inhibiting the uptake of oxLDL.[518] In addition Treg can also directly regulate endothelial cell activation and leukocyte recruitment, independent of their suppressive functions on effector T-cells.[519] Moreover, prolonged hypercholesterolemia resulted in reduction of peripheral and plaque Treg numbers, increased Treg apoptosis and diminished Treg lesional infiltration, but reversal of hypercholesterolemia can restored Treg numbers and stopped lesion growth.[520]

Nevertheless the human atherosclerotic lesions contain only limited Treg numbers (1–5% of all T-cells) given the pro-inflammatory lesion microenvironment, the survival and maintenance of Treg cells would be compromised compared with pathogenic T cells suggesting that protection mediated by Treg is hampered in atherosclerosis.

1.4.4.2.2 Humoral immunity

Despite the complexity of the interactions between the immune and vascular systems, most of the research so far has targeted the cellular aspects of the immune response. Nevertheless, the development of atherosclerosis is also associated with B cell activation suggesting that the humoral immune response might also play a relevant role in this process.

B cells have been found in atherosclerotic lesions in mice[521] and in humans.[522] Moreover it has been shown that patients with atherosclerosis produce antibodies against many antigens, the best characterized are HSP and oxLDL.

HSP are a group of more than 20 molecules categorized in five families according to molecular weight.[523] They are present in all living organisms and represent one of the most conserved protein structures in evolution. HSP have a dual function: inside the cells they help in the assembling and translocation of newly formed proteins as well as in repairing or clearing proteins which have become unstable,[524] and outside the cells they act as stress markers in order to allow the recognition of stressed cells by the immune system.[525]

HSP have been widely associated with atherosclerosis. In the early 1990's, anti-HSP65 antibodies were associated with atherosclerosis.[50] Later, this data would be confirmed in another prospective population-based survey which showed that the same type of antibodies was elevated in subjects with carotid atherosclerosis when compared to those without lesions.[526] More recently, these results have been complemented with the finding of an association between the risk of development of premature atherosclerosis in young males and the level of reactivity of T lymphocytes to human HSP60.[527] Eventually, clinical (cardiovascular) events would also be associated with the presence of these antibodies along with an already known presence of diffuse atherosclerosis.[528] These findings have been thoroughly tested and confirmed in different animal models (e.g. the presence of anti-HSP antibodies in general and anti-HSP60/65 in particular was associated with endothelium injury in rabbits fed with cholesterol-rich diets.[529] Different hypotheses were put forward to explain this association, one of the most popular being the one based on the concept of "molecular mimicry". An immune response against HSP would represent a cross-reaction between HSP produced by endothelial cells subjected to oxidative or other pro-atherogenic stress factors and HSP produced as a response to eventual bacterial and viral infections that happened in the past. In this way, HSP60 expressed by the endothelial cells in response to

any biochemical or haemodinamic stress would become a target of an immune system that has already been sensitized to it on previous infectious events.

However, other possibilities have been put forward to explain such a consistent association of atherosclerosis and anti-HSP antibodies: the protein could become immunogenic due to oxidation or metabolic-induced alterations, other antigens (foreign or self) could interact with HSP60 hence forming a immunogenic complex, the lack of recognition of soluble HSP as a self protein could be due to the fact that it is in most cases an intracellular molecule, and finally the possibility of genetic variations that would keep the immune response active.[530]

These hypotheses are directly or indirectly based on the concept of HSP as an interface between infection and atherosclerosis. In fact, numerous studies have shown a relationship between chronic infection-associated bacteria or viruses and vascular disease. The classic example is the long term infections by Chlamydia pneumoniae and Chlamydia trachomatis which have been associated with an increased rate of atheroma formation and even the sites of infection for the latter (the eye and the fallopian tubes) show fibrosis with the presence of lymphocytes and macrophages in a surprisingly similar way to the development of the atherogenic plaque.[531] Further evidence has been provided in relation to other micro-organisms including Helicobacter pylori, Cytomegalovirus, Epstein-Barr virus amongst others.[532]

The atherosclerotic lesion starts when LDL molecules are trapped in the arterial wall, in the subendothelial extracellular matrix.[56] At this stage, lymphocytic T cells are already present in this lesion, and it was shown that these cells recognize as antigens the oxidized molecules of LDL.[487]

OxLDL are the product of a variety of modifications of both the lipid and protein components of LDL due to the increased concentration of ROS at the subendothelial level. The resulting oxidized phospholipids and aldehyde-modified breakdown fragments of ApoB-100 are not only pro-inflammatory but also are recognized as foreign by the immune system and therefore are highly immunogenic.[533]

In 1989 antibodies to oxLDL (anti-oxLDL) were detected in atherosclerotic patients, experimental animals and in atherosclerotic lesions.[534] Experimental models whereby atherosclerosis suppression was achieved by immunization of hypercholesterolaemic

rabbits[535] or apolipoprotein E-deficient mice,[536] with oxLDL, reinforces the concept that an immune response to oxLDL may modulate atherogenesis.

Both IgM and IgG auto-antibodies to oxLDL are common in humans but their association with cardiovascular disease remains controversially. Although IgG type anti-oxLDL antibodies were positively associated with vascular risk, circulating levels of IgM type anti-oxLDL antibodies have been more frequently linked with reduced vascular risk in humans.[537-539] Moreover, high levels of IgG anti-oxLDL were associated with lipid-rich, fibrous poor plaques (characteristic of vulnerable, rupture-prone plaques), whilst high levels of IgM anti-oxLDL were related with fibrous, lipid-poor plaques containing less macrophages and more extracellular matrix (characteristic of stable plaques with little risk of clinical events).[540]

Further studies are needed to clarify whether oxLDL auto-antibodies are markers of CVD and/or have an active role in either the progression of or protection against the atherogenesis.

In addition to this two main auto-antigens others have been identified, such as β2GP1 and cardiolipins, predominantly in the context of autoimmune diseases.

β2GP1 is a phospholipid-binding protein that is present on platelets, endothelial cells and in human atherosclerotic plaques.[541] Auto-antibodies against β2GP1 have been found in patients with inflammatory disorders, including atherosclerosis, SLE, and antiphospholipid syndrome (APS).

OxLDL interact with this endogenous anti-atherogenic plasma protein to form complexes (oxLDL β2GP1), preventing its uptake by macrophages.[136] OxLDL β2GP1 complexes are found in primary and secondary APS, SLE[136] and chronic nephritis.[542]

The binding of auto-antibodies to these complexes at endothelial level has been reported to be responsible for the endothelial activation and the immune-mediated inflammation.[543] In fact, these immune complexes (antibody towards oxLDL β2GP1) are phagocytised by macrophages much faster, leading to an increased rate of foam cell formation and enhanced atherogenesis.[137] IgG antibodies towards oxLDL β2GP1 are associated with arterial thrombosis in SLE related APS.[543] Ames et al,[544] described a direct association of IgG anti-oxLDL β2GP1 complexes with intima-media thickness (IMT) in primary APS (PAPS), suggesting a potential atherogenic role of these antibodies.

Another aspect is that oxLDL β2GP1 complex binds CRP.[545] Previous studies have revealed that CRP binds phosphorylcholine moieties on oxLDL in what appears a very primitive form of innate immunity.[546] *In vitro* data demonstrated that CRP can bind oxLDL β2GP1 in a ternary complex (CRP-oxLDL β2GP1).[547] This ternary complex was specific for PAPS and predicted by increased IgG-β2GP1, suggesting that an enhanced level of oxLDL might require buffering from both β2GP1 and CRP.[548]

Reason why these complexes and their related auto-antibodies exist as well as their function role remains unclarified.

Anti-cardiolipin (aCL) antibodies are auto-antibodies directed against negatively charged phospholipids and plasma proteins such β2GP1.[549] The aCL antibodies are considered the hallmark of primary APS,[550] a distinct clinical entity characterized by the occurrence of arterial and/or venous thrombosis and recurrent miscarriages. These antibodies have also been detected in the sera of patients with SLE[551] and it has been proposed that they may play a pathogenic role in the accelerated atherosclerosis that afflicts these diseases.[552] The atherogenic mechanism of these antibodies seems to involve the formation of pro-inflammatory IgG- oxLDL β2GP1 complexes.[553]

In immunized mice, aCL and β2GP1 antibodies were detected and associated with the development of atherosclerosis.[554] aCL antibody titres independently predicted IMT of carotid arteries, an acceptable and reliable surrogate marker of sub-clinical atherosclerosis, in subjects with PAPS.[555] In addition, these titres were inversely correlated with the PON1, contributing to the overall increase oxidation found in these patients.[556]

Since the beginning of the 2000´s several others studies support the hypothesis that humoral immunity protects against atherosclerosis. Caligiuri et al,[557]reported that splenectomy increases atherosclerotic lesion development in ApoE($^{-/-}$) mice, whereas B-cell transfer ameliorates atherosclerosis. Likewise, using bone marrow transplantation strategy demonstrated that mice lacking B cells increased lesion growth in Ldlr($^{-/-}$) mice.[558] These observations contribute to the acceptance of a paradigm that, in contrast to other immune-mediated diseases, i.e., RA and SLE, B cells have been assigned a protective role in atherosclerosis.

However, in a major paradigm shift, the two recent studies demonstrated that depletion of B cells in ApoE($^{-/-}$) and Ldlr($^{-/-}$) mice by anti-CD20 monoclonal antibody ameliorated the Western diet-induced atherosclerosis.[559,560]

The atherogenic role of B cells was confirmed in 2012 in studies using hypercholesterolemic B cell activating factor (BAFF) receptor deficient ApoE($^{-/-}$) mice, which reported that atherosclerosis and arterial inflammation was markedly reduced in these animal models.[561,562]

The exact role of B cells in atherosclerosis is not yet fully understood, partly due to incomplete characterization of B cell subsets in humans. B cell subsets have been well recognized in mice, however surface markers that identify B cell subsets differ between mice and humans. While CD5 is a marker of B1 cells in mice, it does not reliably discriminate between B1 and B2 cells in humans.[563,564] In mice both B1 and B2 cells have been shown to play significant roles in atherosclerosis. B1 cells represent a small percentage of B lymphocytes and their major role is in innate immune responses, where they are responsible for the production of the bulk of serum IgM.[565] While B2 cells are predominant population of B cells in spleen and lymph nodes and are the main producers of adaptive IgG antibody after activation by specific antigens and differentiation into plasma cells.[559-561]

Overall, these data contribute to the hypothesis that natural IgM is playing a protective role in atherosclerosis whereas the adaptative IgG response is pro-atherogenic. However the concept of a third subpopulation of B cells with regulatory properties (Breg) was only recently introduced.[566] This B-cell subsets seems to act as immune regulators via IL-10 production, which inhibits pro-inflammatory cytokines and supports regulatory T cell differentiation, although the role of Bregs in atherosclerosis is not yet determined.

1.4.5 Immune response in atherogenesis and HDL

In addition to the multiple anti-atherogenic properties of HDL previously described accumulating evidence also suggests that HDL possesses important function in both innate and adaptive immunity.

Perhaps the interaction between HDL and the immune system longest known is their ability to protection from sepsis caused by the LPS, the endotoxin from gram-negative

bacteria that binds preferentially to HDL preventing LPS to interact with TLRs hence preventing macrophages to release massive amounts of inflammatory cytokines.[567,568]

ApoA-I seems to be a key component responsible for LPS neutralization.[569] Infusion of ApoA-I mimetic peptides into animals with experimental sepsis improves survival preventing defects in vascular function.[570]

Furthermore, HDL also plays a protective role in parasitic infections. The lysis of trypanosomes is mediated by HDL particles that contain ApoL-I and haptoglobin-related protein.[142]

The proteomic analysis of HDL composition identified proteins involved in complement regulation, including C3, C4a, C4b, C9, suggesting that HDL may regulate complement activation and consequently have a role in host defence mechanisms.[86]

In addition to anti-inflammatory properties ApoA-I appears to play an important role in modulating innate immune response. ApoA-I inhibits DCs differentiation from monocytes with a decreased expression of surface costimulatory molecules such as CD1a, CD80, CD86 and increased production of PGE2 and LI-10 (which are known to be DC differentiation inhibitors and/or modulators of DC function).[571] Moreover DCs differentiated in the presence of ApoA-I stimulate T cells to a lesser extent, suggesting that ApoA-I exerts a protective mechanism against the differentiation and activation of DCs.

Likewise, ABC transporter-deficient macrophages resulted in impaired cholesterol efflux to HDL or ApoA-I, leading to accumulate excess free cholesterol in plasma membrane and improvement of lipid rafts formation, which increased TLRs cell surface concentration and enhanced inflammatory responses.[247,572] These studies suggest that HDL and ApoA-I by exerting anti-inflammatory effects by promoting cholesterol efflux via ABCG1 and ABCA1 attenuated TLR activation (Figure 27).

Figure 27. Role of HDL and ApoA-I in regulating immune responses by promoting cholesterol efflux via ATP-binding cassette transporters (ABCG1 and ABCA1) with consequent attenuation of toll like receptor (TLR) signaling, which is activated by the increased lipid raft. From Weber et al, 2011.[573]

Besides cholesterol depletion from lipid rafts downregulates signalling pathways in several immune cells it also disturb the antigen-presenting capacity.[574]

It has been shown that MHC class II molecules are localized in lipid rich microdomains on the surface of APCs, the integrity of this lipid rafts are important for the antigen presentation to T and B cells.[575,576] In fact the number of MHC class II molecules present in this lipid rich microdomains are important because it provide a mechanism for localized concentration of MHC class II-peptide complexes on the surface of APCs which decrease the amount of the antigen required for T or B activation.

Recently, Wang et al[577] showed that HDL and ApoA-I reduced cholesterol and MHC class II protein content in lipid rafts, suggesting that cholesterol efflux from APCs to HDL and ApoA-I inhibits antigen presentation and consequently T cell activation.

Despite these significant function of HDL in the immune system, an humoral response towards HDL has not been fully explored and only few studies have addressed the importance anti-HDL (aHDL) antibodies and its potential pathogenicity.[556,578,579]

1.5 Aims of the thesis

We hypothesized that antibodies towards HDL and/or its components are associated with modifications in the anti-atherogenic functions of HDL and might represent a new mechanism of atherogenesis.

The goals of this thesis are to identify and characterize the humoral response towards HDL components and to evaluate the possible mechanism that may contribute to the modifications of the anti-atherogenic properties of HDL.

The specific aims are:

1) To identify the presence of antibodies towards HDL components in patients with vascular diseases. For this purpose we used different cohorts: a) patients with SLE, a disease characterised by an enhanced humoral activity with a high prevalence of cardiovascular events; b) diseases associated with a higher atherosclerotic burden such as CAD, cerebrovascular disease and diabetes.

2) To determine whether these antibodies have any biologic effect. For this purpose aHDL antibodies were isolated from patients and introduced in several experiments design to determine if their presence interfered with HDL anti-oxidative and anti-inflammatory properties.

3) To investigate whether drugs acting on plasma lipids and lipoproteins in different fashions could alter the antibody profile.

2. METHODS

The following chapter describes the methods that were used throughout the thesis, according to the methodology and the order that they are referred to in the results chapter All the information about the subjects in the different studies (healthy controls and patients) and the selection criteria for the clinical trials will be described in their respective results chapter.

2.1 Immunologic-related methods

2.1.1 Enzyme-Linked Immunoabsorbent Assays (ELISAs)

2.1.1.1 Performance parameters /optimization of assays

To determine the appropriate assays conditions for the ELISAs assays described in the following sections, of each step/component was performed using a check board. This enable the optimization of two components simultaneously and the maintenance of all the other reagents constant. Optimization of the assays included the determination of the type of plate, assay temperature, duration of incubation, coating concentration, blocking buffer, standards, controls and serum dilutions (always in triplicates), secondary antibody concentration and signal detection. The optimization of the assay was performed using negative controls, with high and low analyte concentrations. Optimum concentrations were selected based in the maximal desired optical density and minimum background activity e ratio.

All assays were validated by the inclusion of internal quality control samples of known activity. Results were expressed as a percentage of the positive control present in each plate after subtraction from the background in the uncoated half of the plate. Exceptions are mentioned. Inter/intra plate coefficients of variation were always less than 10 %. Sera from 150 healthy subjects was used for standardisation of the methods for determination of titres of the different antibodies. Values >3 standard deviations (SD) above the mean of healthy age and sex matched controls were considered to be positive.

2.1.1.2 Anti-high density lipoproteins (aHDL) IgG and IgM antibodies

IgG and IgM aHDL antibodies were measured by ELISA using a 96-well microtitre plates (Polysorp, Nunc, VWR, Portugal) half-coated with 20 µg/mL (IgG) or 10 µg/mL (IgM) human HDL (Sigma-Aldrich, Sintra, Portugal) in 70 % ethanol up to evaporation at 37ºC. The plates were then blocked (non-specific binding) with the addition of 100 µL/well of 1 % or 2 % (w/v)

of bovine serum albumin (BSA from Sigma-Aldrich) - 10 mM phosphate buffer saline (PBS from Sigma-Aldrich) pH 7.4 for IgG or IgM antibodies, respectively, over one hour at 37 ºC. The unbound blocking agent was removed by washing the plates four times with PBS. Next, positive and negative controls and samples were diluted (1:100) in the respective blocking buffer (1 % BSA - 10 mM PBS pH 7.4 for IgG or 2 % BSA - 10 mM PBS pH 7.4 for IgM) and added in both halves of the plate for one hour at 37 ºC. The unbound antibodies were removed by repeating the washing step. Secondary antibodies (Sigma-Aldrich) alkaline phosphatase (AP) conjugated anti-human IgG (1:1000 in blocking buffer) or anti-human IgM (1:5000 in blocking buffer) were added for one hour at 37 ºC. Following three washes with PBS and three washes with bicarbonate (BIC) buffer pH 9.8 the colorimetric reaction was performed by the addition of 100 µL/well p-nitrophenyl phosphate (pNPP from Sigma-Aldrich) 1:5000 in BIC buffer, an AP substrate and allowed the reaction to proceed for one hour at 37 ºC. Absorbance of the resultant yellow colour was measured by Biotrak II plate reader (Amersham Biosciences) at 405 nm.

2.1.1.3 Anti-apolipoprotein A-I (aApoA-I) IgG antibodies

IgG aApoA-I antibodies were measured by half-coating a 96-well plate (Polysorp) with 10 µg/mL human ApoA-I (Sigma-Aldrich) in 70 % ethanol up to evaporation at 37ºC. Blocking (non-specific binding) was performed using 100 µL/well 1% BSA - 10 mM PBS pH 7.4 for 1 hour at 37ºC following four washes with PBS. Serum samples, positive and negative controls (1:200 in blocking buffer) and standards were added to each half of the plate for 1 hour at 37ºC. The washing step was repeated and the secondary antibody AP conjugated anti-human IgG (1:1000 in blocking buffer) added for 1 hour at 37ºC. After washing three times with PBS and three times with BIC buffer pH 9.8 the plates were incubated with 100 µl pNPP (1:5000 in BIC buffer) for 1 hour at 37 C. Absorbance of the resultant yellow colour was measured at 405 nm.

Initially the titres of aApoA-I antibodies were expressed as the percentage of the positive control present in each plate after subtraction from the background in the uncoated half of the plate. After chapter 3, section 3.2 the aApoA-I antibody titres were converted in µg/mL, as determined by the standard curve present in each plate. This curve was prepared with six standards of human monoclonal aApoA-I antibody in a concentration range of

0.001-0.04 μg/mL. In both assays the inter and intra plate coefficients of variation were always less than 10%.

2.1.1.4 Anti-paraoxonase 1 (aPON1) IgG antibodies

Human PON1 (Abnova, Tebu-bio, Portugal) was adsorbed on half-microtitre plates by incubating 100 μL/well at a concentration of 1 μg/mL prepared in 70 % ethanol up to evaporation at 37ºC. The plate was blocked with 1 % BSA - 10 mM PBS pH 7.4 for 1 hour at 37 ºC and washed four times with PBS. Serum samples, positive and negative controls (1:300 dilutions in blocking buffer) were added in both halves of plate for 1 hour at 37 ºC. After repeating the washing step, the secondary antibody AP conjugated anti-human IgG (1:1000 in blocking buffer) was added for 1 hour at 37 ºC. To remove the unbound conjugate the plate was washed three times with PBS and three times with BIC buffer pH 9.8. The plate was incubated with 100 µL pNPP (1:5000 in BIC buffer) and after 1 hour the absorbance was measured at 405 nm.

2.1.1.5 Anti-apolipoprotein A-II (aApoA-II) and C-I (aApoC-I) IgG antibodies

Ninety-six well plates (Polysorp) were half-coated up to evaporation at 37ºC with 10 µg/mL human Apo A-II (Sigma-Aldrich) or 2 µg/mL human Apo C-I (Sigma-Aldrich) in 70 % ethanol. The plates were blocked with 1 % BSA - 10 mM PBS pH 7.4 for 1 hour at 37 ºC. Samples and positive control were diluted in blocking buffer (1:100) and added in both halves of the plates for 1 hour at 37 ºC. The washing step was repeated and the anti-human IgG conjugated with AP (1:1000 in blocking buffer) added to each well for 1 hour at 37ºC. Following three washes with PBS and three washes with BIC buffer pH 9.8 the plate was incubated with 100 µL pNPP (1:5000 in BIC buffer) for 1 hour at 37 C. Absorbance of the resultant yellow colour was measured at 405 nm.

2.1.1.6 3-Nitrotyrosine (3-NT)

Serum concentration of 3-NT were determined by ELISA using commercial kits (Oxis Research, Foster City CA, USA) in accordance with the manufacturer's instructions. Standard curves were performed in each plate, using samples with known 3-NT concentrations included in the kit and the results were expressed as nM. Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.1.7 Soluble vascular cell adhesion molecules (VCAM-1) and intracellular cell adhesion molecules (ICAM-1)

Soluble adhesion molecules (VCAM-1 and ICAM-1) levels were measured by ELISA using commercial kits (R&D systems, Citomed, Portugal), according to the manufacturer's instructions. Standard curves were performed in each plate, using samples with known VCAM-1 or ICAM-1 concentrations, respectively included in each kit and being the results expressed as ng/mL. Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.1.8 Vascular endothelial growth factor (VEGF)

Levels of VEGF from supernatant of cell cultures were quantified by ELISA using commercial kits (R&D systems, Citomed, Portugal), in accordance with the manufacturer's instructions. Standard curves were performed in each plate, using samples with known VEGF concentrations included in the kit and the results expressed as pg/mL. Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.1.9 High sensitivity C-reactive protein (hs-CRP)

Serum concentration of hs-CRP were assessed by ELISA using commercial kits (Biosupply Ltd, Bradford, UK), in accordance with the manufacturer's instructions. Standard curves were performed in each plate, using samples with known hs-CRP concentrations included in the kit and the results expressed as µg/mL. Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.1.10 Serum amyloid A (SAA)

SAA levels were evaluated by ELISA using commercial kits (Europa Bioproducts, Ely, UK), according to the manufacturer's instructions. Standard curves were performed in each plate, using samples with known SAA concentrations included in each kit and being the results expressed as µg/mL Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.1.11 Oxidised low density lipoprotein-beta 2 glycoprotein-I complex (oxLDL β2GP1)

oxLDL β_2GP1 complex levels were determined by ELISA using commercial kits (Corgenix, Bloomfield, Colorado, USA), according to the manufacturer's instructions. Standard curves were performed in each plate, using samples with known oxLDL β_2GP1 concentrations

included in each kit and being the results expressed as units/mL. Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.1.12 Asymmetric dimethylarginine (ADMA)

Serum concentration of ADMA were quantified measured by ELISA using commercial kits (DLD, Diagnostika GmbH, Hamburg, Germany), in accordance with the manufacturer's instructions. Standard curves were perform in each plate, using samples with known ADMA concentrations, included in each kit and being the results expressed as µM. Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.2 Immunoturbidimetric immunoassay

2.1.2.1 Apolipoprotein A-I

Serum concentration of ApoA-I was determined by a immunoturbidimetric immunoassay using commercial kits (Randox, Irlandox - Laboratorios Quimica Analitica Lda, Porto, Portugal) in accordance with the manufacturer's instructions. The test is based on the reaction of a sample containing ApoA-I (calibrator or serum sample) and specific antiserum to form an insoluble complex which can be measured turbidemetrically at 340 nm by RX Daytona (Randox). ApoA-I concentration was determined using a WHO/IFCC reference standard[580] and the results expressed as mg/dL. Repeatability and accuracy of the assay was ensured by the inclusion of internal quality control sample.

2.1.3 Antibody isolation

2.1.3.1 aHDL and aApoA-I IgG antibodies isolation in a 96-well microtitre as solid phase

aHDL antibodies were isolated by solid phase adsorption using microtitre plates (Polysorb) half-coated overnight at 4 °C with 20 µg/ml human HDL in 70 % ethanol. Blocking was performed using 1 % BSA - 10 mM PBS pH 7.4 for 1 hour at 37 ºC. The plate was washed four times with PBS. Serum samples positive for IgG aHDL antibodies (1:50 in blocking agent) were added in quadruplicate in both half of the plate for 1 hour at 37 ºC. The washing steps were repeated and then 250 µL of 100 mM glycine buffer (pH 2.0) were added. The content of each quadruplicate well was aspirated to a tube which contained 70 µL of Tris buffer 1 M pH 9.0 for neutralization. Afterwards, each fraction was dialyzed against PBS and then lyophilised (Christ Alpha 1-Z, B. Braun Biotech International). The procedure for the aApoA-I antibody isolation was overall similar the only difference being that the antigen used to coat

the plate was 10 µg/mL of human ApoA-I and the serum samples were selected based on their highly positive activity for the antigen. In each fraction, the titres of aHDL or aApoA-I antibodies were tested by ELISA following the protocol described in sub-chapter 2.1.1.2 and 2.1.1.3 respectively.

2.1.3.2 aHDL IgG antibodies isolated by immunoaffinity chromatography

aHDL antibodies were isolated using an HiTrap NHS- activated HP (1 mL) column. The ligand, human HDL (10 mg) was dissolved in coupling buffer (0.2 M NaHCO3, 0.5 M NaCl pH 8.3). The column was embedded in isopropanol to preserve the stability of the activated medium and immediately prior the coupling of HDL the column was washed with six column volumes (6 mL) of 1 mM ice-cold HCl using a syringe of 1 mL at a flow rate of 1 mL/min (1/2 drop/sec). Following the addiction of the HDL solution, the column was sealed and left to stand for 45 minutes at room temperature. To deactivate any excess active groups that had not coupled to the ligand and the non-specifically bound ligands, the column was washed with 6 mL of 0.5 M ethanolamine, 0.5 M NaCl pH 8.3 (buffer A) following 6mL of 0.1 M acetate, 0.5 M NaCl pH 4 (buffer B) and again with the same volume of buffer A. After this, the column was sealed and left standing for 30 minutes at room temperature. The washes were then restarted using buffer B interspersed with buffer A. In the end of the second wash with buffer B the column was washed with 10 mM phosphate buffer NaH_2PO_4, 2.7 mM KCl, 137 mM NaCl pH 7.4. After this step the column was ready for use. If it was not used immediately, it was stored, after applying a storage buffer consisting of 0.05 M Na_2HPO_4, 0.1 % NaN3 pH 7.

Before applying the samples, the column was conditioned with 10 mL of binding buffer (20 mM Tris pH 7.5). Serum samples were diluted in equal volume of the binding buffer and filtered through a Millex GV 0.22 µm filter. The diluted serum was then applied into the column using a 1 mL syringe and passed over at least six times at room temperature. The unbound antibodies were removed by washing the column with 10 mL of 20 mM Tris pH 7.5 and further 10 mL of 20 mM Tris, 500 mM NaCl pH 7.5. Antibodies retained by the HDL-column were eluted with 10 mL of 0.1 M glycine pH 2.5 into tubes containing 70 µL of the neutralization buffer (Tris 1 M pH 8.8). After elution of the antibodies at low pH, the column was washed with 10 mL of 20 mM Tris pH 8.8 and then with 10 mL of 20 mM pH 7.5 to reconditioned the column.

Absorbance (280 nm) of the collected fractions (1 mL) was measured and the peak fractions were dialyzed against PBS and tested for the presence of aHDL antibodies by the ELISA procedure described above (sub-chapter 2.1.1.2). The fractions positive for the antibodies were pooled, concentrated by evaporation under nitrogen and stored at -4 ºC until tested for their biologic activity.

2.2 Biochemistry-related methods

2.2.1 Paraoxonase 1 (PON1) activity

Serum PON1 activity was measured according to Eckerson et al,[581] with some modifications. Paraoxon (1mM) (Sigma-Aldrich) was freshly prepared in 300 µL of 50 mM glycine buffer containing 1 mM CaCl (pH 10.5) and then was incubated at 37 ºC with 10 µL of serum for 10 min in 96 well plates (PolySorp). Para-nitrophenol formation was monitored at 412 nm. Enzyme activity was calculated with a molar extinction coefficient of 18.290 Mcm-1 and expressed as U/L, which is defined as 1µmol of p-nitrophenol generated per minute per litter under assay conditions. All samples were tested in triplicate. Inter/intra plate coefficients of variation were always less than 10%.

2.2.2 Total anti-oxidant capacity (TAC)

TAC was assessed by measuring the capacity of a sample to scavenge peroxynitrite ($ONOO^-$) formed by the reaction between $O_2^{\bullet -}$ and NO• released from a solution of 3-morpholino-sydnonimine hydrochloride (SIN-1). The assay was performed in a Greiner 96 F White plate using an ABEL-41M2 antioxidant test kit (Knight Scientific, Plymouth, UK) which uses Pholasin (a photoprotein that produces light in the presence of free radicals and certain oxidants), as a probe. The light emission was measured every 54 seconds for 1 hour at 25 ºC using an Anthos Zenyth 1100/3100 multimode detector. The antioxidant capacity of the sample is defined as the time maximum light was emitted i.e. the time taken to consume the available antioxidants. The time to achieve peak luminescence (peak time) was converted into Vitamin E analogue (VEA) equivalent units (µM), using six solutions of 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid with known concentration to generate a standard curve in each plate.

2.2.3 Nitric oxide metabolites

NO• has a short biological half-life (10–20 milliseconds) that cannot be measured directly, so must be inferred by indirect methods. Estimation of stable NO• end products, nitrite (NO_2^-) and nitrate (NO_3^-), are widely used as indicators of the effective availability of NO•.[582] Therefore, these metabolites were determined using a modified Griess reaction, following the reduction of nitrate to nitrite using nitrate reductase and NADPH as previously reported.[583] Briefly, the assay was performed in a standard flat-bottomed 96-well plates half divided for simultaneous measurement of NO_2^- and NO_3^- concentration. Serum was diluted one in four with PBS pH 7.4, and 200 µl of this solution was ultra-filtered by centrifugation at 10000 g for 1 hour, using 10000 kD molecular weight filters (Ultrafree-MC, Millipore, VWR). Only clear and colourless filtrates were tested. Fifty µL/well of PBS (blank), standard or serum sample were added in duplicate to both halves of the plate. In one of the halves, 4 µL of nitrate reductase (Sigma-Aldrich) and 10 µL of NADPH (Sigma-Aldrich) were added for the reduction of NO_3^- to NO_2^-, giving a final concentration of 6.3 U/L and 550 µM respectively. Following 2 hours of incubation at room temperature, Griess reaction was initiated by adding equal volumes of 2 % sulfanilamide (Sigma-Aldrich) in 5 % H3PO4 and 0.2 % N-(1-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich) in water, mixed just prior to use. After 10 minutes of incubation at room temperature the absorbance was read at 540 nm and the levels were expressed as µM.

2.2.4 Total HDL cholesterol, HDL2 and HDL3

(This quantifications were made by Firmina Lebre, Técnica Especialista de Análises Clínicas of FCM).

Total HDL cholesterol (HDL-C), HDL2 and HDL3-cholesterol were determined by a precipitation assay using the QUANTILOP HDL (HDL2/HDL3) test reagent. The test consists of the addition of the precipitation reagents containing polyethylene glycol (PEG) in different concentrations and pH to the serum.

Reagent A precipitates VLDL and LDL fractions, while Reagent B precipitates along with VLDL, LDL, HDL2 subclass. Therefore, after centrifugation of the solution precipitated with Reagent A the supernatant contains all the HDL subclasses whereas the centrifugation of the solution precipitated with Reagent B, creates a supernatant containing only the HDL3 subclass. The cholesterol content of these supernantants was quantified using the CHOD-

PAP enzymatic test reagent at 500 nm . The content of HDL2 cholesterol was obtained by subtracting the HDL3 to total HDL.

2.3 Functional assays in vitro

2.3.1 Inhibition of paraoxonase 1 (PON1) activity by the antibodies isolated from patient's serum

2.3.1.1 aHDL and aApoA-I IgG antibodies isolated from patients systemic lupus erythematosus (SLE)

Dose dependent inhibition was performed by incubating 50 µL of human HDL as a source of PON1 with 50 µL of aHDL or aApoA-I antibodies isolated from patients with SLE (descried above in sub-chapter 2.1.3.1) for 1 hour at 37 °C. Irrelevant human IgG (Sigma-Aldrich) at the same concentration of HDL was used as controls and glycine buffer. After 1 hour of incubation, PON1 activity was measured as described in sub-chapter 2.2.1. A dose dependent inhibition test was performed by incubating doubling dilutions (2, 4, 8 and 16 fold) of HDL (starting at 200 µg/mL). All experiments were run in triplicate.

2.3.1.2 aHDL IgG antibodies isolated from patients with atherosclerosis-associated clinical events: ischemic stroke (IS) and coronary artery disease (CAD)

Dose dependent inhibition assays of PON1 activity were performed by incubating human HDL (100 µg/mL) with PBS pH 7.4 (basal) or aHDL IgG antibodies isolated from patients with IS and CAD (pool) (descried in sub-chapter 2.1.3.2). Irrelevant human IgG was used as control at the same concentration of the antibodies tested. After 1 hour of incubation, PON1 activity was assessed as described in sub-chapter 2.2.1. All experiments were run in triplicate and the results were expressed as a percentage of the effect on PON1 activity in basal conditions.

2.3.2 In vitro exposure of human umbilical vein endothelial cells (HUVECs) to aHDL antibodies

Confluent monolayers of HUVECs seeded in 24-well tissue culture plates were incubated for 16 hours at 37 °C in 5 % CO2, in different conditions: M199 medium (Sigma-Aldrich) supplemented with 1% fetal calf serum (basal); human HDL (1.6 mg/mL) with or without aHDL antibodies (50 µg/mL) isolated from serum of patients (descried in sub-chapter 2.1.3.2), a non-specific human IgG (50 µg/mL), or human nonoclonal aApo A-I antibody

(30 µg/mL). HDL and the antibodies were incubated for 1h at 37ºC before cell addiction. After washing, the cells were incubated further for 4 hours in the basal or stimulated state with 10 ng/mL TNF-α. Following three washes with PBS, cells were gently dislodged by brief trypsinization (0.025 % trypsin and 2 mM EDTA in PBS) for 3 minutes at room temperature. Trypsin was neutralized with PBS and the cells were centrifuged at 12000 rpm for 5 minutes. The supernatants were colleted and stored at -20ºC for VEGF quantification as described in sub-chapter 2.1.1.8. The cells were stained with a fluorescein-conjugated mouse monoclonal anti-human VCAM-1 (R&D system) added for 30 minutes at 4°C and then washed again with FACS buffer (PBS containing 0.1% BSA and 0.01% sodium azide) to remove the unreacted anti-VCAM-1 reagent. The expression of VCAM-1 was measured as fluorescence intensity in a FACSCalibur (Becton Dickinson) flow cytometer. Ten thousand cells were analyzed per sample by using Cell Quest Software. Data was expressed as the percentage of basal VCAM-1 expression positive cells. All experiments were done in triplicate.

Cell viability was determined by a lactate dehydrogenase release assay and found to be >90% in all experiments.

2.4 Statistical analysis

Data was expressed as means ± SD, unless otherwise stated. Variation between groups was compared by ANOVA, or Kruskal-Wallis test for normal and non-normal distributed variables, respectively. Likewise, a t-test or Mann-Whitney test was used to compare individual groups depending on variable distribution. Relationships between variables were assessed by Spearman correlation. Multiple regression was used to assess independence of relationships after log- transformation, when appropriate, to normalise distributions.

All reported probability values are two-tailed, with values of $p < 0.05$ being considered statistically significant. Statistical analysis and graphical illustration was carried out using the GraphPad Prism software, version 5 (GraphPad Software Inc., San Diego, USA). Determination of independent predictors was done using the SPSS version 21 (SPSS Inc., Chicago, USA)

3. HUMORAL RESPONSE TOWARDS HDL COMPONENTS AND ITS ASSOCIATION WITH MODIFICATIONS OF THE ANTI-ATHEROGENIC PROPERTIES OF HDL

The presence of antibodies against lipoproteins suggests a humoral response against some lipoprotein components, independently of a possible underlying auto-imune condition.

This thesis deals mostly with cross sectional studies, that limit causuality assumption, but represents the baseline from which prospective population-based studies may be developed.

To enhance the chance of detecting these antibodies we initially explored their presence in SLE a systemic disease characterised on the one hand by an enhanced and pleitropic humoral response and on the other by a higher cardiovascular risk identified as increased atherogenesis. The confirmation of their presence with a high prevalence and biologic activity allowed the obvious extention towards the general population with known, common atherosclerosis risk factors but without immune-mediated comorbidities.

3.1 aHDL and aApoA-I antibodies in systemic lupus erythematosus (SLE)

SLE is a chronic autoimmune systemic disease with a wide spectrum of clinical manifestations ranging from skin and mucosal lesions to severe injuries in the central nervous system, kidneys and other organs. It is characterized by systemic inflammation and by the presence of auto-antibodies against a large variety of auto-antigens.[584]

SLE is much more frequent among women than men, with a ratio of approximately 9:1, although this ratio can change in different age groups. Additionally, ethnic origins is thought to be a major factor in SLE onset, as African-American, Afro-Caribbean, Asian Indian, Polynesian and Chinese women are are more likely to develop disease than European descents.[585,586]

The diagnosis of SLE is based on clinical and laboratory criteria. For the purpose of classification the most widely used are the American College of Rheumatology (ACR) criteria, proposed in 1971 and revised in 1982 and again in 1997.[587,588]

Genetic, hormonal, environmental and infectious factors are involved in the pathogenesis of SLE.[589] Furthermore multiple immune system defects have been described in SLE patients, including B cell hyperactivity and increased auto-antibody production, abnormal T cell activation, aberrant cytokine production and enhanced apoptosis.[590-592]

Currently, more than one hundred auto-antibodies have been described in SLE, these include autoantibodies directed against a multiplicity of nuclear, cytoplasmic, cell

membrane, phospholipid-associated, blood cells, endothelial cells, and nervous system antigen, plasma proteins, matrix proteins, and miscellaneous antigens.[593]

Over the past 50 years, improved patient survival in SLE has allowed the emergence of CVD as a major cause of morbidity and mortality.[594] However, the traditional cardiovascular risk factors does not explain the increased CVD burden in SLE, and therefore it has been suggested that disease-specific factors, such as prolonged steroid treatment, chronic inflammation and renal disease, could be more relevant.[595-597]

Furthermore, an atherogenic lipid profile, characterized by elevated levels of VLDL, TG and lower levels of HDL has been reported in SLE.[598] Indeed, decreased levels of HDL and its main protein component, Apo A-I, have been shown to be an important feature in the development of atherosclerosis in SLE.[599,600] These findings might be relevant considering that HDL is a particular lipoprotein with different atheroprotective functions.

The occurrence of autoantibodies against plasma lipoproteins and their constituents has been addressed in SLE but with major emphasis on anti-LDL and/or its oxidized form, with the possibility of cross-reactivity between these autoantibodies and aCL antibodies .[601]

Very few studies have addressed the importance of the humoral response towards HDL and the potential pathogenesis of these antibodies in SLE.[556,578,579]

3.1.1.1 Aim

This study aimed at confirming the presence of a humoral response towards HDL in SLE and to assess whether Apo A-I could be a specific target. We have also explored the relationship between aHDL and aApo A-I antibodies and the anti-oxidant and anti-inflammatory functions of HDL in SLE, to determine a possible association with disease activity and damage.

3.1.1.2 Patients and methods

Patients

SLE patients attending the Lupus Outpatients Clinic of University College London, London, UK and controls of similar age and sex, recruited amongst healthy staff members and students attending the rheumatology department were invited to participate in a study assessing the relevance of new markers of atherosclerosis in SLE. Exclusion criteria for SLE patients were secondary APS, a positive aCL and/or lupus anticoagulant test even in the absence of thrombosis or miscarriage and the use of lipid-lowering drugs. Ninety-eight

consecutive patients fulfilling at least four of the ACR revised criteria[588] for the classification of SLE were considered, of whom 21 were excluded: 16 had at least one of the exclusion criteria and 5 declined to participate.

Exclusion criteria for controls were diabetes, hyperlipidaemia, hypertension, kidney, liver, heart or lung disease, and intake of lipid-lowering drugs. A total of 64 healthy subjects were invited to participate but 14 were not considered: 8 met one or more of the exclusion criteria and 6 were excluded in order to achieve a frequency match by sex and age with the study group.

The study was therefore carried out on 77 SLE patients and 50 controls, after approval from the local ethics committees in agreement with the revised Declaration of Helsinki and with written consent being given by all participants.

Data regarding steroid dose, immunosuppressant drugs use and anti-platelet agents were available for all SLE patients. Disease-related damage was determined at the time of serum sampling using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SLICC/ACR DI).[602] The disease activity was assessed using the British Isles Lupus Assessment Group (BILAG-2004) index: patients scored Grade A or B in any system were categorised as having active disease and patients scored Grade C, D or E in all systems were categorised as having inactive disease.[603] Serum from patients and controls were kept at –80ºC until analysis. Demographics and clinical data of patients and controls are shown in Table 6.

Methods

Serum IgG aHDL and aApo A-I antibodies titers and levels of soluble VCAM-1 and ICAM-1 were measured by ELISAs. PON1 activity, TAC and nitric oxide metabolites (NO_2^- and NO_3^-) were tested as described in the methods chapter. Lipid profile (total cholesterol, HDL, LDL and TG) were determined by standard enzymatic techniques in the hospital when sample was collected. Statistical analysis was performed as described in the previous chapter.

3.1.1.3 Results

IgG aHDL and aApo A-I antibodies, PON1 activity and plasma lipids

Patients with SLE showed lower HDL and higher TG, LDL and total cholesterol than controls (Table 6).

Table 6. Demographic characteristics and clinical and serological data of healthy controls (CTRL) and patients with systemic lupus erythematosus (SLE). Data are presented as mean ± SD.

	CTRL (n = 50)	SLE (n = 77)	P value
Age, years	36.56 ± 13.17	39.61 ± 11.75	0.2
N. (%) female/male	45 (90) / 5 (10)	68 (88.3) / 9 (11.7)	0.7
Global score (BILAG)	NA	4.5 ± 3.8	-
Medication			
Steroids, n. (%)	NA	52 (67.5)	-
Steroid dosage (mg/day)	NA	6.74 ± 3.34	-
Hydroxycloroquine, n. (%)	NA	32 (41.6)	-
On immunosuppressant treatment, n. (%)	NA	37 (48.0)	-
Serological data (mg/dL)			
Total cholesterol	139.6 ± 55.9	201.0 ± 41.6	<0.0001
TG	91.3 ± 58.9	133.7 ± 92.4	0.006
LDL-C	76.7 ± 35.7	111.3 ± 35.0	<0.0001
HDL-C	66.6 ± 25.3	57.5 ± 13.3	0.02

Abbreviations: BILAG:British Isles Lupus Assessment Group; TG: Triglycerides; NA Not applicable. Data are presented as mean ± SD. P value represents the statistical comparison between the two groups.

Mean IgG aHDL and aApo A-I antibodies were higher in SLE patients than healthy controls (Figure 28 A and B) and positively correlated (r = 0.64, P<0.0001) in the SLE population. The prevalence of positive titres of IgG aHDL antibodies were 59.7% in SLE patients and 8% in healthy controls, whilst for aApo A-I antibodies were 50.6% in SLE patients and 10% in healthy controls.

HDL-C levels showed an inverse correlation with IgG aHDL (r = -0.32, p=0.02) and IgG aApoA-I (r = -0.41, p=0.001).

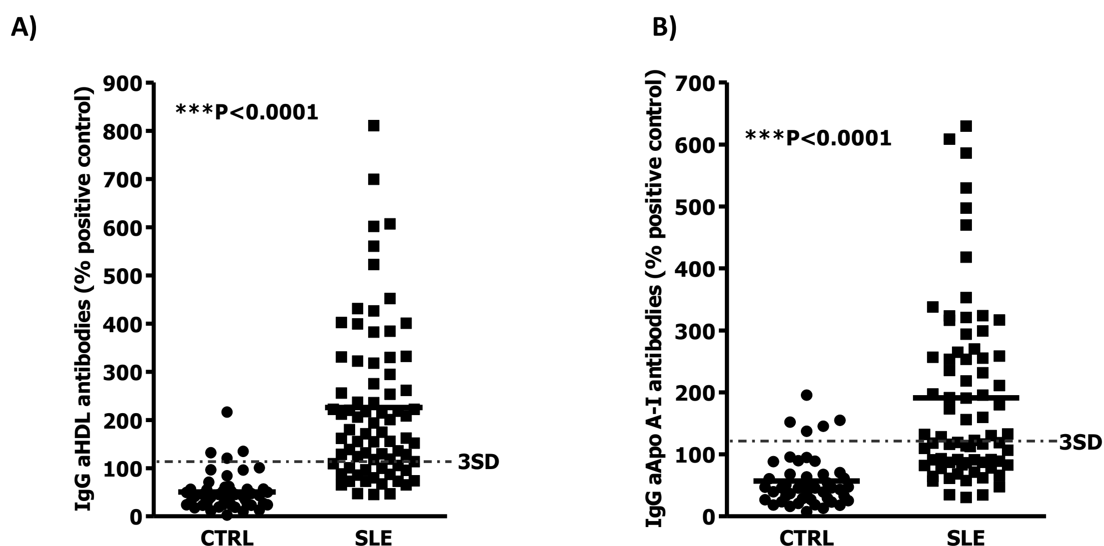


Figure 28: Levels of IgG aHDL (A) and IgG aApoA-I antibodies (B) in healthy controls (CTRL) and patients with systemic lupus erythematosus (SLE) Bars show the means.

Mean PON1 activity was lower in SLE than controls (P<0.0001) (Table 7) and negatively correlated to IgG aHDL (r = -0.34, P=0.002) and IgG aApo A-I titres (r = -0.31, P=0.006) (Figure 29 A and B).

Table 7: Biological variables (oxidation and endothelial dysfunction markers) measured in healthy controls (CTRL) and patients with systemic lupus erythematosus (SLE).

	CTRL (n = 50)	SLE (n = 77)	P value
PON1 activity (U/L)	303.60 ± 60.38	185.00 ± 86.37	<0.0001
TAC (VEA equivalents units μM)	6773 ± 1343	5448 ± 2519	0.001
NO_2^- (μM)	10.97 ± 6.60	20.71 ± 11.81	<0.0001
NO_3^- (μM)	20.75 ± 13.92	37.94 ± 19.51	<0.0001
NO_2^- + NO_3^- (μM)	31.42 ± 15.48	58.52 ± 25.95	<0.0001
VCAM-1 (ng/mL)	558.00 ± 91.67	816.80 ± 357.90	<0.0001
ICAM-1 (ng/mL)	280.40 ± 48.27	346.20 ± 106.40	0.0008

Abbreviations: PON1: paraoxonase 1; TAC: Total antioxidant capacity; NO_2^-: nitrite; NO_3^-: nitrate; 3-NT: 3-nitrotyrosine; VCAM-1: vascular cell adhesion molecule; ICAM-1: intracellular adhesion molecule. Data are presented as mean ± SD. P value represents the statistical comparison between the two groups.

Current treatment did not affect the titres of IgG aHDL and aApoA-I antibodies or PON1 activity in the SLE group.

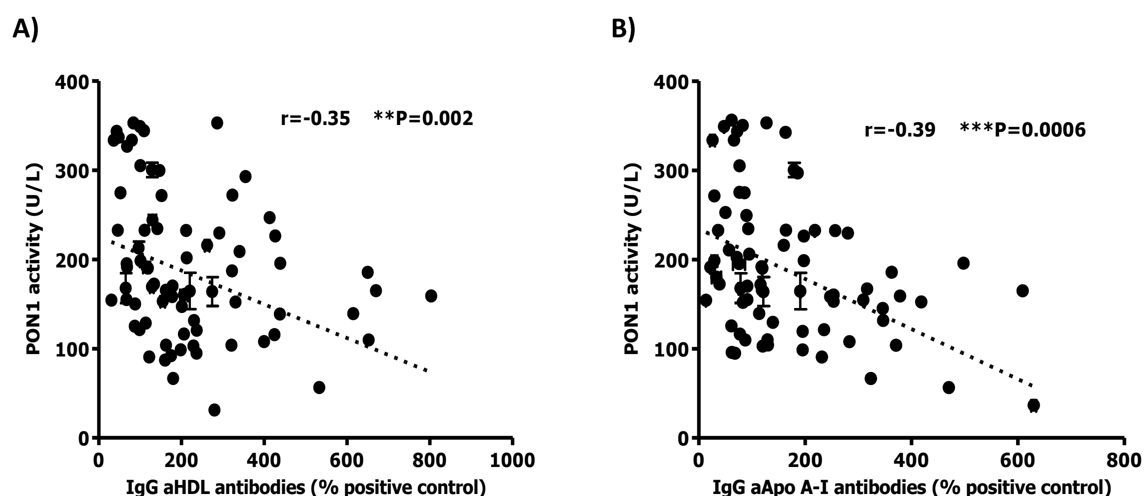


Figure 29: Correlation (Spearman's rank test) between PON1 activity (U/L) and IgG aHDL levels (A) and IgG aApoA-I levels (B) in patients with systemic lupus erythematosus (SLE).

In a regression model including HDL-C or PON1 activity as the dependent variables and age, smoking, current steroid dose, current immune suppressor dose, other lipid proflie measurements, IgG aHDL, aApoA-I, SLICC/ ACR and BILAG as independent variables, higher IgG aApoA-I titre were independently associated with lower levels of HDL-C (t = -3.04, p = 0.004) and decrease PON1 activity (t = -2.35, p = 0.02).

Total antioxidant capacity (TAC) and nitric oxide metabolites (NO_2^- and NO_3^-)

TAC was lower in patients with SLE than in healthy controls (p=0.001) (Table 7) and inversely correlated with to the titres of IgG aHDL (r = -0.43, p = 0.0005) and IgG aApoA-I (r = -0.47, p = 0.0001). There was a positive association with PON1 activity (r = 0.32, p = 0.0009) (Figure 30).

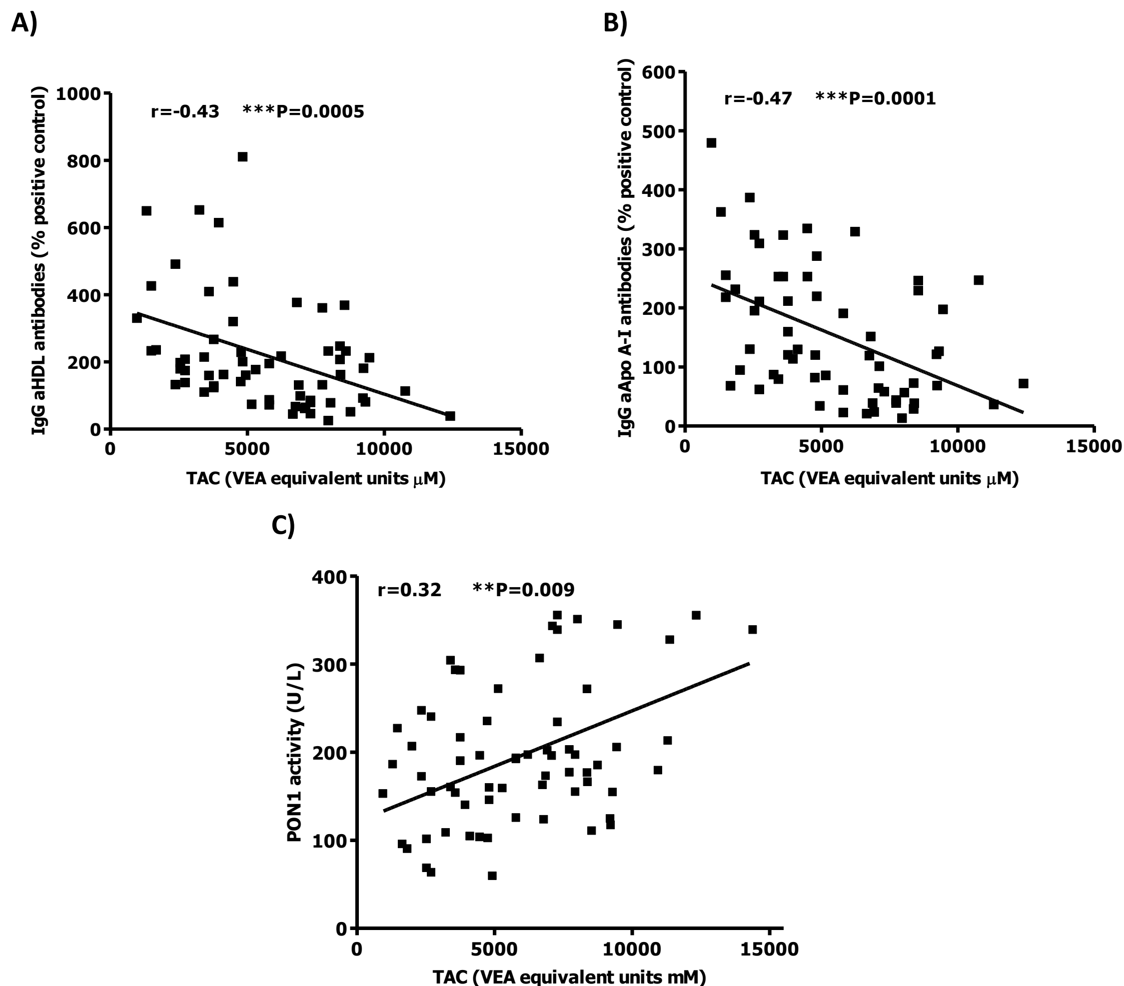


Figure 30: Correlation (Spearman's rank test) between the total antioxidant capacity (TAC) expressed in Vitamin E analogue (VEA) equivalent units (μM) and IgG aHDL (A), IgG aApoA-I (B) antibodies titres and PON1 activity (C) in patients with systemic lupus erythematosus (SLE).

As both NO_2^- and NO_3^- levels were higher in patients with SLE compared to healthy controls (p<0.0001) (Table 7) for the association analyses the sum of both metabolites was used. In SLE patients, NO_2^- + NO_3^- levels showed a direct correlation with IgG aHDL (r = 0.33, p = 0.009) and IgG aApoA-I (r = 0.42, p = 0.008) antibodies and an inverse correlation with PON1 activity (r = 0.32, p = 0.0009).

Soluble vascular adhesion molecules (VCAM-1) and soluble intracellular adhesion molecules (ICAM-1).

Mean levels of VCAM-1 and ICAM-1 were higher in patients with SLE compared to healthy controls ($p<0.0001$ and $p = 0.0008$, respectively (Table 7). In the SLE group, IgG aHDL antibody titres independently predicted ICAM-1 ($t = 3.51$, $p = 0.001$) whereas IgG aApoA-I and $NO_2^- + NO_3^-$ independently predicted VCAM-1 ($t = 3.31$, $p = 0.002$ and $t = 2.27$, $p = 0.02$, respectively). VCAM-1 ($r = -0.39$, $p = 0.004$) and ICAM-1 ($r = -0.32$, $p = 0.02$) inversely correlated with PON1 activity but no relationship was observed with TAC.

Relationship between serological and clinical data

SLE patients with a higher damage index had elevated IgG aHDL and IgG aApoA-I titres (Figure 31 A) and lower PON1 activity (Figure 31 A). Patients with active disease (BILAG) showed higher titres of IgG aHDL, IgG aApoA-I and lower PON1 activity than patients with inactive disease (p= 0.03, p= 0.03 and P= 0.002, respectively) (Figure 31 B).

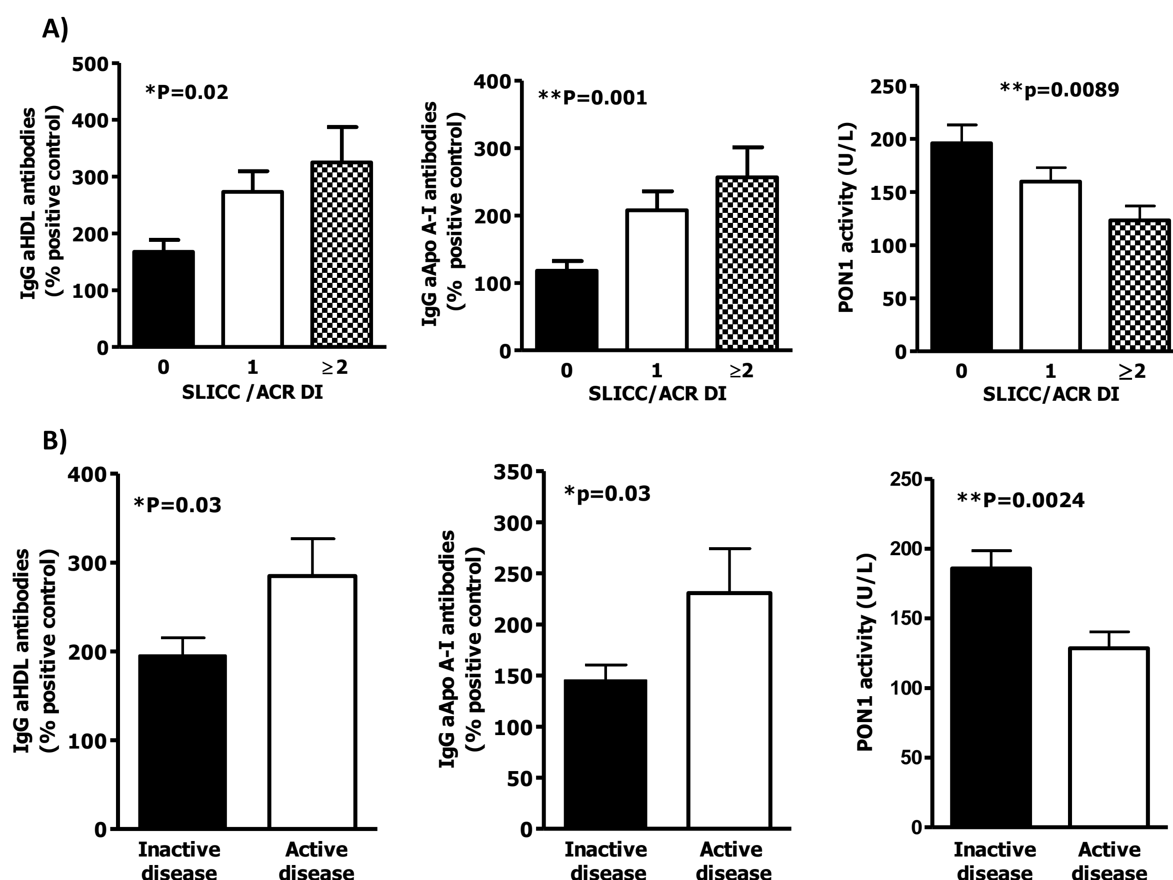


Figure 31. Relationship between the titres of IgG aHDL, aApoA-I antibodies and PON1 activity with SLICC/ACR damage index (A) and disease activity (BILAG score) (B) in patients with systemic lupus erythematosus (SLE) (Kruskal-Wallis test – Anova).

Mean NO_2^-+NO_3^- was associated with an increase in the SLICC/ACR DI ($p = 0.02$), but no association was found with disease activity (Figure 32 A).

TAC was not associated with either disease activity or damage. VCAM-1 levels were associated with an increased SLICC/ ACR DI ($p = 0.006$) and disease activity ($p = 0.03$) (Figure 32 B and C), but no associations were found between ICAM-1 levels and clinical data.

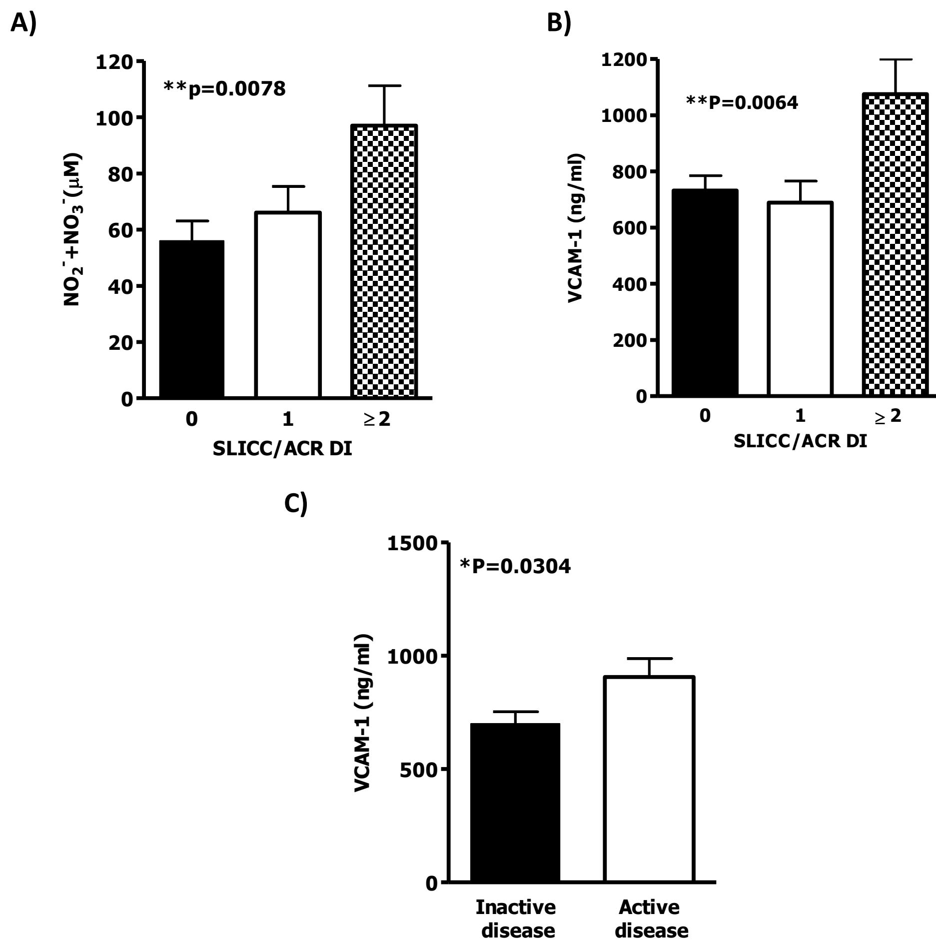


Figure 32. Relationship between the NO_2^-+NO_3^- (A) and VCAM-1 (B, C) levels with damage (SLICC/ACR DI) and disease activity (BILAG score) in patients with systemic lupus erythematosus (SLE) (Kruskal-Wallis test – Anova).

In a regression model including SLICC/ ACR as the dependent variable and age, smoking, current steroid dose, current immune suppressor dose, HDL-C, IgG aHDL, aApoA-I, PON1 activity, NO_2^-+NO_3^-, VCAM-1, ICAM-1 and BILAG as independent variables, higher IgG aHDL antibodies titres and , NO_2^-+NO_3^- levels were independently associated with high SLICC/ACR damage index ($t = 2.44$, $p = 0.02$ and $t = 2.61$, $p = 0.01$, respectively).

3.1.1.4 Discussion

This study confirms the presence of antibodies towards HDL, and its main protein component ApoA-I, in patients with SLE. They are associated with decreased PON1 activity, reduced TAC (suggesting a lower resistance to oxidation of the plasma), endothelial activation (elevated NO_2^- + NO_3^-, VCAM-1 and ICAM-1), and with an increase in damage and disease activity. Altogether, these findings suggest that IgG aHDL antibodies increase the risk of oxidative stress, therefore contributing to the accelerated atherogenesis present in SLE.

In this cohort, titres of IgG aHDL and aApoA-I antibodies strongly correlate, suggesting that ApoA-I might be one of the key antigens for aHDL antibodies. This does not exclude the possibility of other potential targets within the HDL complex.

Patients with SLE have increased levels of TG, LDL-C, and total cholesterol and lower HDL-C levels when compared with controls.[598] In this patients, IgG aApoA-I antibody titres were independently associated with HDL-C levels, suggesting that quantity as well as quality of HDL may be influenced by them.

The presence of IgG aHDL and aApo A-I antibodies may have clinical implications as their relationship with the SLICC/ACR DI indicates. This finding is further highlighted by the lower PON1 activity in patients with higher disease activity. PON1 is the enzyme that accounts for most of the capacity of HDL to prevent oxidation of LDL, and consequent reduction of foam cell formation. By interfering with PON1 function, aHDL and aApoA-I may tilt the oxidant/anti-oxidant balance towards oxidative stress. The activity of this enzyme has already been associated with particular SLE-related features such as lupus nephritis.[604]

NO• plays a central role in regulating vascular tone, but its overproduction may contribute to tissue injury, by increasing the vascular permeability, generating toxic-free radicals such as $ONOO^-$, and inducing cytotoxicity.[261] Belmont et al,[605] reported overexpression of iNOS in periods of active disease. NO• has a short half-life and its metabolites, including nitrite and nitrate, are widely used as indicators of the effective availability of NO•.[606] Our study confirms elevated plasma NO• concentration in patients when compared with healthy controls.[605,607]

Under normal conditions, NO• inhibits vascular muscle cell proliferation, platelet aggregation and monocyte adhesion to the endothelium. However, free radicals such as $ONOO^-$, when excessively generated by the reaction of NO• with superoxide, induce

modifications in proteins, lipids and DNA which may increase the immunogenicity of intracellular antigens, leading to a break in immune tolerance.

Furthermore, the overproduction of free radicals and the excess NO• concentration primes the vascular endothelium for subsequent injury. Here we report for the first time that NO• is in a positive relationships with IgG aHDL and aApoA-I antibodies and in a negative one with PON1 activity, highlighting how these changes may reflect clinical damage (SLICC/ACR DI).

Oxidative stress plays a vital role in the pathogenesis of atherosclerosis and its complications. TAC reflects the capacity of serum to resist oxidation and has been defined as a global measure that takes into account the overall antioxidant defence of serum.

In the present study, TAC of patients with SLE was significantly lower than the control group, and IgG aHDL and aApoA-I antibodies were negatively associated with TAC. Moreover the positive correlation between TAC and PON1 activity suggests that IgG aHDL and aApoA-I may decrease TAC by an inhibitory effect on PON1. Since PON1 inhibits oxidation of phospholipids, thus preventing $ONOO^-$-mediated lipid peroxidation, the reduction of PON1 activity, as seen in SLE patients, would further increase the overall oxidative state showed by a reduction in TAC.

The interaction of leucocytes with the vascular endothelium is pivotal to the inflammatory process, and is mediated, amongst others, by adhesion molecules, such as VCAM-1 and ICAM-1, which participate in atherogenesis by promoting monocyte adhesion to the endothelium and subsequent accumulation in the arterial intima. In the above process, HDL inhibits the expression of cell surface adhesion molecules by activated endothelial cells.[213] Levels of soluble adhesion molecules have been shown to correlate with various cardiovascular risk factors including low HDL-C.[608]

Our study showed that soluble VCAM-1 levels correlate with low HDL levels and with disease activity, being significantly higher during active disease. Soluble ICAM-1 levels did not reflect disease activity. Whilst the variations in VCAM-1 are in agreement with previous studies,[609-611] ICAM-1 levels have generated contradictory results, with studies reporting a positive correlation between s-ICAM-1 levels and disease activity,[612] and others failing to find significant differences when compared with healthy controls, or even an association with disease activity.[609,613] This difference was addressed by Cybulsky et al,[614] who showed that VCAM-1 deficiency significantly diminishes early foam cell lesion formation in the aorta of

mice deficient in the LDL receptor (Ldlr -/-), suggesting that VCAM-1 may play a role in preventing the initiation of atherosclerosis. However, ICAM-1 deficiency did not influence early foam cell lesion formation, either alone or when combined with VCAM-1 deficiency. Despite the up-regulation of both adhesion molecules in atherosclerotic lesions, VCAM-1 seems to play a more important role in the initiation of atherosclerosis.[614]

The association between VCAM-1 and ICAM-1 levels and IgG aHDL and aApoA-I antibodies and PON1 activity was demonstrated by the first time in this study. Moreover the IgG aHDL and aApoA-I antibodies titres independently predicted higher levels of ICAM-1 a VCAM-1 levels, respectively, suggesting that the presence of the antibodies may induce the synthesis these molecules by inhibiting HDL.

In conclusion, by interfering with the anti-atherogenic properties of HDL, IgG aHDL and aApoA-I are associated with an enhanced oxidative stress and a higher SLE-related atherosclerotic risk. Given the cross-sectional nature of the study, disease activity and damage cannot be causally linked with the markers under study: their prognostic value with regards to SLE-related vascular disease should be assessed prospectively in further studies. At this point we asked ourselves whether this was a SLE-specific phenomenon or was it present also in the non autoimmune setting

3.2 Anti-HDL and anti-ApoA-I antibodies in non auto-immune patients with atherosclerosis-related clinical events

3.2.1 Coronary artery disease (CAD) and ischemic stroke (IS)

CAD (or atherosclerotic heart disease) is the end result of the accumulation of atheromatous plaques within the walls of the coronary arteries and is one of the most relevant clinical features of atherosclerosis. Although without the same strong association IS can also be related to an increased atheroma burden in the arteries supplying blood to the brain.

Despite major advances in the understanding of the nature of the atherosclerotic plaque and the phenomenon of plaque rupture, together with the proliferation of coronary care units, angioplasty and bypass surgery, thrombolysis, pharmacologic treatment for

hypercholesterolemia and hypertension (e.g. statin and angiotensin-converting enzyme (ACE) inhibitors) and a tremendous emphasis on lifestyle modification, CAD and IS continue to be major causes of mortality and morbidity throughout the world.[1,2]

Epidemiologic and clinical studies show an inverse correlation between HDL-C and an increased incidence of cardiovascular diseases[354] and the importance of HDL is now more recognised not just for preventing atheroma formation but also for stabilising plaques, therefore preventing rupture and ultimately thrombosis.[239,447-449]

The existence of a possible humoral response towards the HDL complex was found in patients with autoimmune disease like SLE where enhanced atherogenesis and overactive immune response are prominent features.

The suspicion that antibodies against HDL might be present outside the context of autoimmune diseases, led us to test this hypothesis in non auto-immune patients with atherosclerosis-related clinical events.

3.2.1.1 Aim

This study aimed to identify the presence of antibodies towards HDL and/or some of its components in patients with atherosclerosis-related clinical events such as CAD and IS and to evaluate their association with endothelial dysfunction and with some of the HDL anti-oxidative properties.

3.2.1.2 Patients and methods

Patients

One-hundred and eleven consecutive patients (56 CAD and 55 IS) with atherosclerosis-associated clinical events, followed at the Internal Medicine outpatients clinic of Curry Cabral Hospital from November 2007 to September 2008 were invited to participate in this study. Inclusion criteria were: routine follow up appointment, for patients with at least a vascular event confirmed by CT scan (IS) or coronary angiography (CAD) in the last 5 years, without evidence of active systemic inflammation (normal erythrocyte sedimentation rate - ESR) and hs CRP < 1.0 mg/dL. Exclusion criteria included renal or hepatic dysfunction (serum creatinine < 1.2 mg/dL and ALT/AST < 2x reference value), infection, neoplastic disease, decompensated cardiac insufficiency and a thrombotic event of any kind in the previous 3 months.

Of the initial cohort, 16 patients with CAD and 15 with IS were excluded: 11 CAD and 12 IS to match both groups for age (the oldest patients were excluded), 5 did not have CAD confirmed and 3 had had a cerebral haematoma and not IS. Forty patients with CAD and 40 with IS were definitely enrolled in the study. Ten patients with CAD also had an IS and 5 patients with cerebral events had cardiac insufficiency, but no coronary disease was confirmed. Two patients with IS had atrial fibrillation.

Healthy subjects used as controls were recruited during the same period of time amongst family and friends of the patients. Demographic data of patients and controls, medical history including current medication and other clinical data are summarized in Table 8.

All subjects signed consent forms approved by the ethics committees of the Hospital and the study was carried out according to the revised declaration of Helsinki.

Blood samples were collected after a 12h fast and were centrifuged at 3000 xg at 4ºC for 10 min to obtain serum. Serum from patients and controls were kept at –80°C until assayed.

Methods

Serum IgG /IgM aHDL, IgG aApoA-I, IgG aPON1, IgG aApoA-II and aApoC-I antibodies titres and levels of soluble VCAM-1, ICAM-1 and 3-NT were measured by ELISAs. PON1 activity, TAC and nitric oxide metabolites (NO_2^- and NO_3^-) levels were also tested as described in methods chapter. Lipid profile (total cholesterol, HDL-C, LDL-C and TG) were determined by standard enzymatic techniques in the hospital when sample was collected. Statistical analysis was performed as described in methods.

3.2.1.3 Results

Characteristics of the study subjects

The characteristics of the study subjects are shown in Table 8. Patients groups were more likely to present with co-morbidities such hypertension (65-75%), type 2 diabetes (20-30%) and dyslipidemia (22.5-25%) and to be on medication than controls.

Table 8: Demographic characteristics and clinical and serological data of healthy controls (CTRL) and patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS).

	CTRL (N=40)	CAD (N=40)	IS (N=40)	P value
Age (years)	67 ± 12	78 ± 7	69 ± 12	‡0.0004
Female sex (%)	50	50	50	0.89
Medical History, N (%)				
Hypertension	4 (10)	26 (65)	30 (75)	<0.0001
Type 2 diabetes	1 (2.5)	12 (30)	8 (20)	0.004
Dyslipidemia	2 (5)	10 (25)	9 (22.5)	0.04
Medication, N (%)				
ACE inhibitors	1 (2.5)	12 (30)	7 (17.5)	0.004
ARB	0	2 (5)	2 (5)	0.36
Beta-Blocker	0	7 (17.5)	5 (12.5)	0.01
Calcium-channel blocker	0	7 (17.5)	4 (10)	0.02
Diuretics	2 (5)	14 (35)	9 (22.5)	0.004
Anti-platelet agents	0	13 (32.5)	12 (30)	0.0004
Statin	0	9 (22.5)	5 (12.5)	0.007
Lipid levels (mg/dL)				
HDL-C	56.7 ± 22.2	34.9 ± 13.5	37.4 ± 13.3	<0.0001
LDL-C	82.1 ± 30.9	100.0 ± 36.3	112.3 ± 42.1	0.002
Total cholesterol	153.4 ± 51.7	140.7 38.1	164.7 ± 57.9	0.11
Triglycerides	95.2 ± 45.6	110.3 ± 37.6	137.2 ± 65.6	0.003

Abbreviations: ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker. NA: not applicable. Data are presented as mean ± SD. P value represents the statistical comparison between the three groups, Kruskall Wallis with Dunn's post hoc. ‡ Statistical comparison between CTRL and CAD patients

IgG aHDL, aApoA-I and aPON1 antibodies in patients with CAD and IS

Mean titres of IgG aHDL, aApoA-I and aPON1 antibodies were higher in both patient groups, when compared to healthy controls (Figure 33 A-C; p < 0.0001).

No differences were observed for the remaining antibodies tested (IgG aApoA-II, aApoC-I and IgM aHDL) (Table 9).

Table 9: Antibodies titres measured in healthy controls (CTRL) and in patients with atherosclerosis-associated clinical events coronary artery disease (CAD) and ischemic stroke (IS).

	CTRL	CAD	IS	P value
IgG aHDL (% p. control)	87.5 ± 26.9	184.5 ± 81.3	182.6 ± 115.4.5	<0.0001
IgM aHDL (% p. control)	43.3 ± 29.6	46.8 ± 65.1	42.3 ± 62.5	0.11
IgG aApoA-I (µg/mL)	0.49 ± 0.22	1.19 ± 0.74	1.14 ± 0.98	<0.0001
IgG aApoA-II (% p. control)	154.6 ± 133.3	178.2 ± 119.4	165.2 ± 120.5	0.70
IgG aApo C-I (% p. control)	334.8 ± 318.7	372.9 ± 353.6	336.4 ± 310.4	0.94
IgG aPON1 (% p. control)	87.2 ± 57.8	163.5 ± 114.5	211.5 ± 162.9	<0.0001

Abbreviations: aHDL: anti-high density lipoprotein antibodies; aApo A-I: anti-apolipoprotein A-I antibodies; aApo A-II: anti-apolipoprotein A-II antibodies aApo C-I: anti-apolipoprotein C-I antibodies; aPON1: anti-paraoxonase 1 antibodies; Kruskall Wallis with Dunn's post hoc.

The prevalence of positive titres of IgG aHDL were 97.5% for patients with CAD, 95% for patients with IS and 45% in healthy controls, whilst for aApo A-I antibodies were 60% and 77.5% for patients with CAD and IS, respectively and 25% in healthy controls. The prevalence of positive titres of IgG aPON1 in patients with CAD was 40% and 32.5% in patients with IS while in healthy controls was 12.5%.

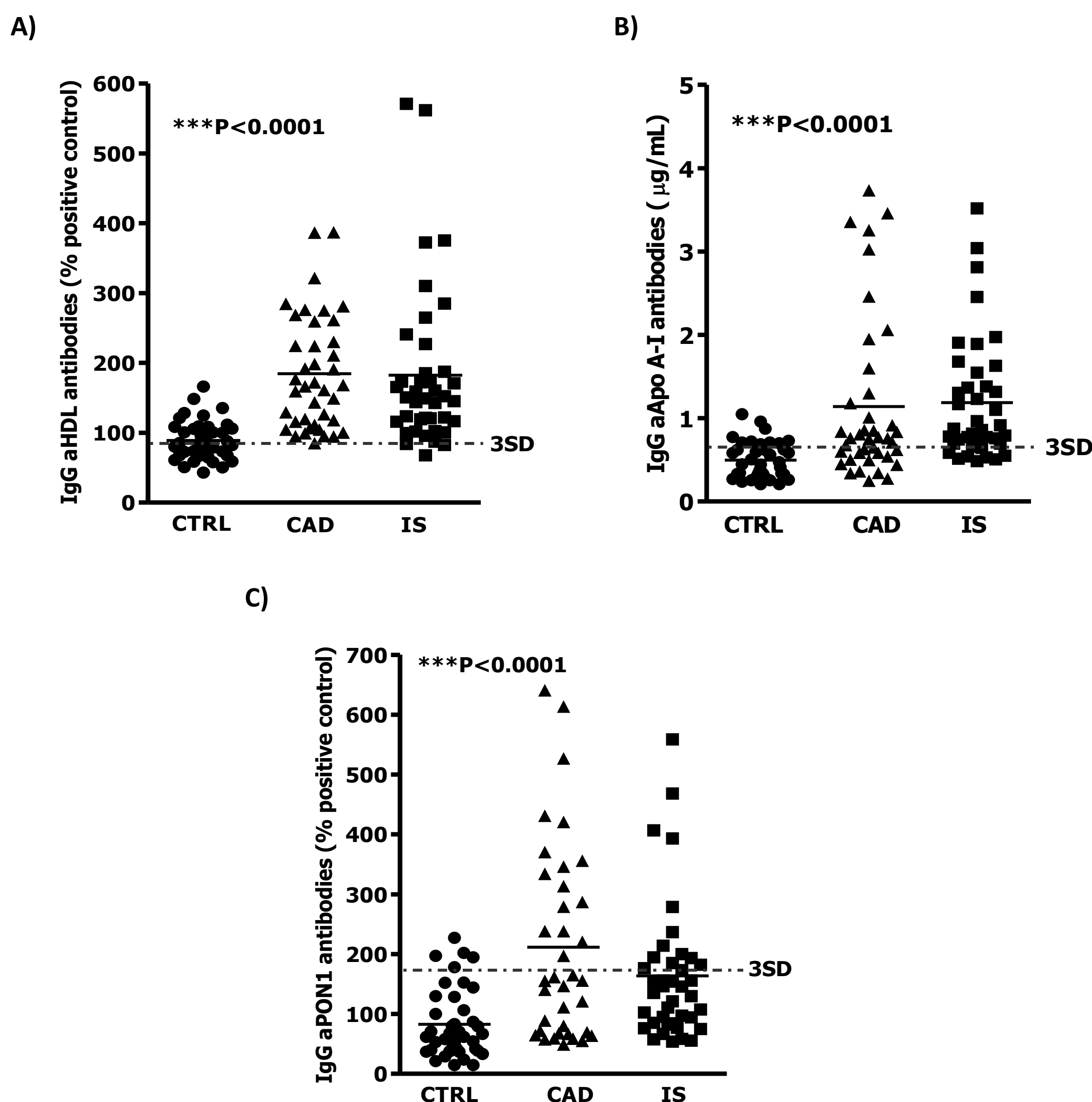


Figure 33. Levels of IgG aHDL (A), aApoA-I (B) and aPON1 (C) antibodies in healthy controls (CTRL) and patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS). Bars show the means. Kruskall Wallis test.

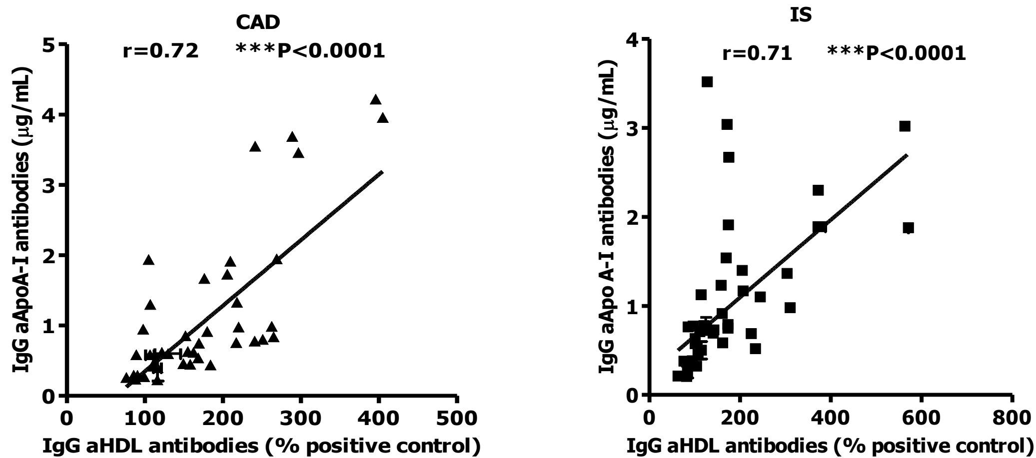
IgG aHDL directly correlated with the IgG aApoA-I titres ($p < 0.0001$ in CAD and IS) and IgG aPON1 titres $p < 0.0001$ but only patients with CAD (Figure 34 A, B).

Co-morbidities such hypertension, diabetes melitus and dyslipidemia were not associated with differences between antibodies titres in the patients with CAD and IS, or

with different treatment modalities. There were also no differences when we compared patients with and without hypertension regardless of the groups.

Although total cholesterol did not differ between patients groups and healthy controls, LDL-C and TG levels were higher and HDL-C levels were lower in both patients groups, when compared to healthy controls, as expected (Table 8). No correlation was found between HDL-C levels the presence of IgG aHDL, aApoA-I or aPON1 antibodies.

A)



B)

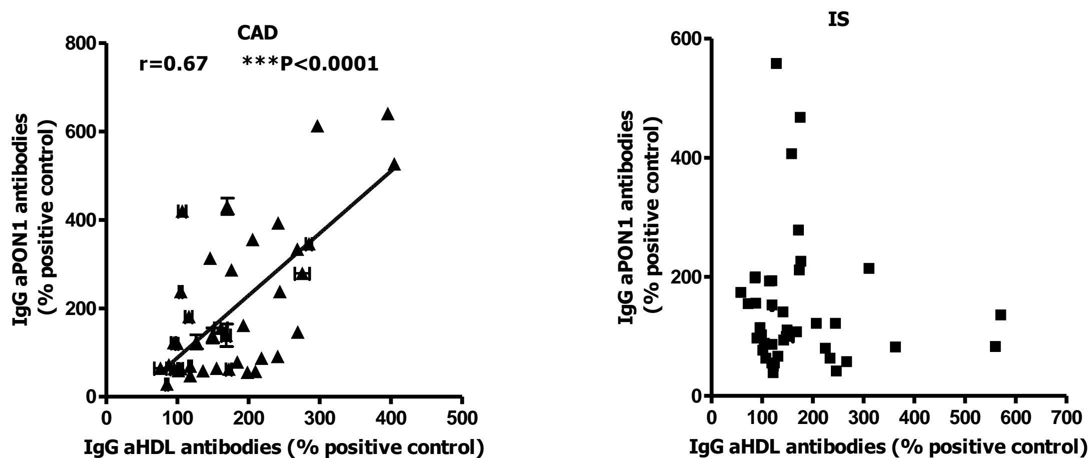


Figure 34. Relationship between the titres of IgG aHDL and IgG aApo A-I (A) and aPON1 antibodies (B) in both group of patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS).

PON1 activity and association with antibody titres

PON1 activity was lower in both groups of patients when compared to healthy controls ($p<0.0001$, Table 10) and negatively correlated with the titres of aApo A-I antibodies (CAD $p = 0.006$ and IS $p = 0.01$) and aPON1 antibodies (CAD $p = 0.007$ and IS $p = 0.0004$) (Figure 35 A, B).

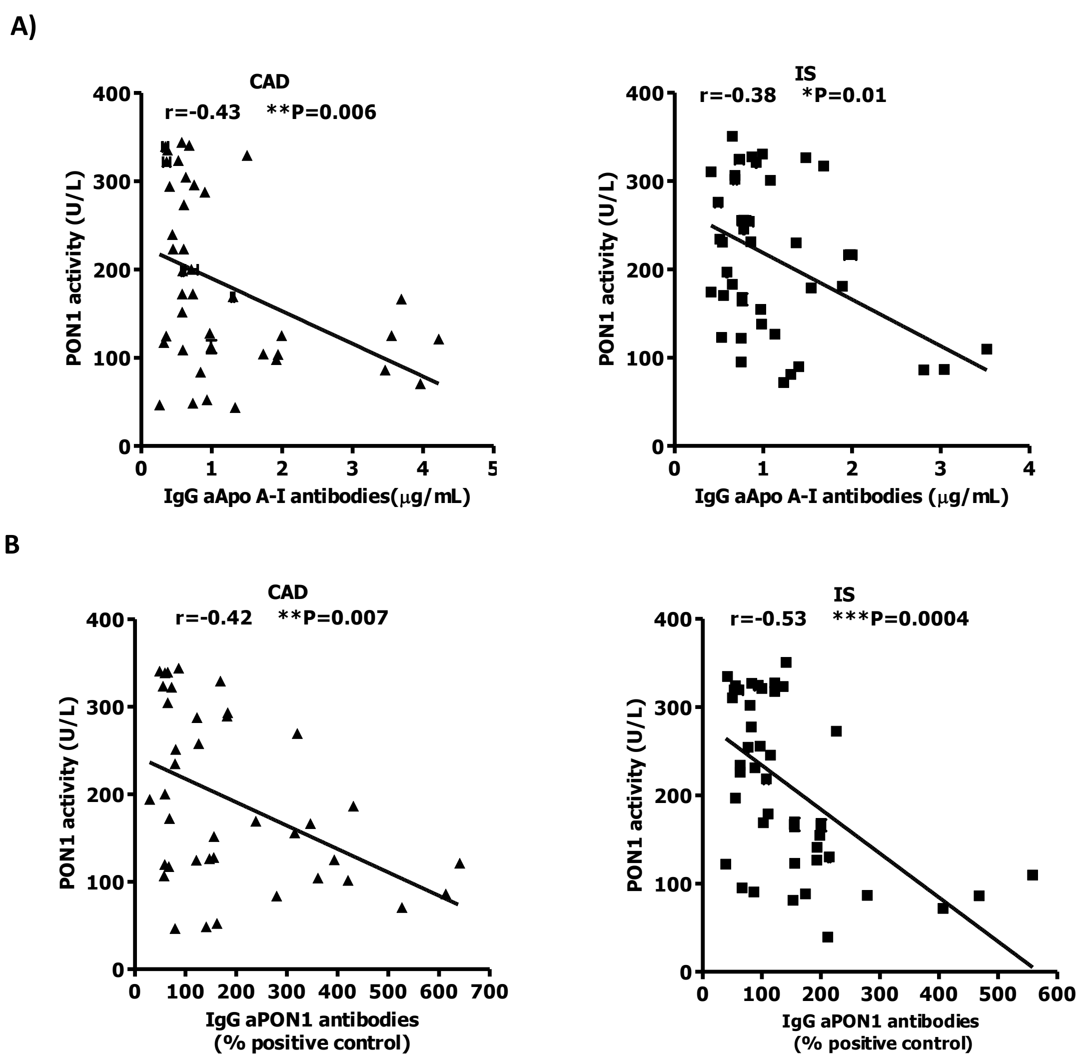


Figure 35. Correlation (Spearman's rank test) between PON1 activity (U/L) and IgG aApoA-I (A) and aPON1 antibodies (B) in both groups of patients with atherosclerosis-associated clinical events coronary artery disease (CAD) and ischemic stroke (IS).

No difference was observed in the mean levels of TAC (Table 10) between both groups of patients and healthy controls.

When age, HDL-C, LDL-C, TAC and IgG aHDL, aApoA-I and aPON1 antibodies titres were entered as independent variables In a regression model with PON1 activity as the dependent variable for the CAD group IgG aApoA-I antibodies titres negatively predicted PON1 activity ($t = -2.91$, $p = 0.006$); when a similar regression model was applied to the IS group IgG aPON1 antibodies titres and high HDL-C levels resulted as independent negative ($t = -4.65$, $p = 0.0001$) and positive ($t = 4.16$, $p = 0.0001$) predictors of PON1 activity respectively.

Table 10: Biological variables (oxidation and inflammation markers) measured in healthy controls (CTRL) and in patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS).

	CTRL	CAD	IS	P value
PON1 activity (U/L)	275.7 ± 76.7	181.2 ± 97.3	190.6 ± 88.0	<0.0001
TAC (VEA equivalents units μM)	8.1 ± 1.6	8.9 ± 2.8	8.0 ± 2.0	0.13
NO_2^- (μM)	13.4 ± 8.8	8.6 ± 4.2	9.6 ± 6.1	0.006
NO_3^- (μM)	25.6 ± 15.9	42.8 ± 33.5	16.5 ± 11.4	0.006
3-NT (nM)	16.2 ± 3.9	21.2 ± 8.3	19.2 ± 5.7	0.002
VCAM-1 (ng/mL)	589.8 ± 158.2	1534.0 ± 792.5	1057.0 ± 547.6	<0.0001
ICAM-1 (ng/mL)	297.6 ± 69.1	462.4 ± 156.4	347.6 ± 76.9	<0.0001

Abbreviations: PON1: paraoxonase 1; TAC: total antioxidant capacity; NO_2^-: nitrite; NO_3^-: nitrate; 3-NT: 3-nitrotyrosine; VCAM-1: vascular cell adhesion molecule; ICAM-1: intracellular adhesion molecule. Kruskall Wallis with Dunn's post hoc: NO_3^- (CAD vs IS p<0.001); VCAM-1 (CAD vs IS p <0.001); ICAM-1 (CAD vs IS p<0.001).

Endothelial dysfunction parameters have distinct profiles between patients groups

Mean NO_2^- levels were decreased in both patient groups when compared with controls ($p = 0.006$, Table 10) and with no significant difference between CAD and IS. Mean NO_3^- levels were higher in CAD patients when compared to IS patients ($p < 0.001$) and healthy controls ($p = 0.006$, Table 10). Additionally, 3-NT, VCAM-1and ICAM-1 levels were increased in both patient groups, when compared to healthy controls (Table 10, $p = 0.002$, $p < 0.0001$, $p < 0.0001$, respectively, Table 10). VCAM-1 and ICAM-1 levels were higher in CAD patients than in IS patients ($p < 0.001$).

In CAD patients the levels of NO_3^- were directly correlated with IgG aHDL ($p = 0.02$), aApoA-I ($p = 0.006$) and aPON1 ($p = 0.02$) antibodies (Figure 36 A-C) whereas in IS patients the levels of NO_2^- were inversely correlated with IgG aApoA-I ($p = 0.0001$) and aPON1 ($p = 0.02$) antibodies.

The levels of 3-NT were directly correlated with IgG aHDL and aApoA-I antibodies ($r = 0.36$, $p = 0.02$) and NO_3^- levels ($r = 0.41$, $p = 0.01$) in CAD patients, whereas in IS patients 3-NT levels were positively correlated with IgG aApoA-I antibodies ($r = 0.47$, $p = 0.003$) and inversely correlated with NO_2^- levels ($r = -0.37$, $p = 0.01$).

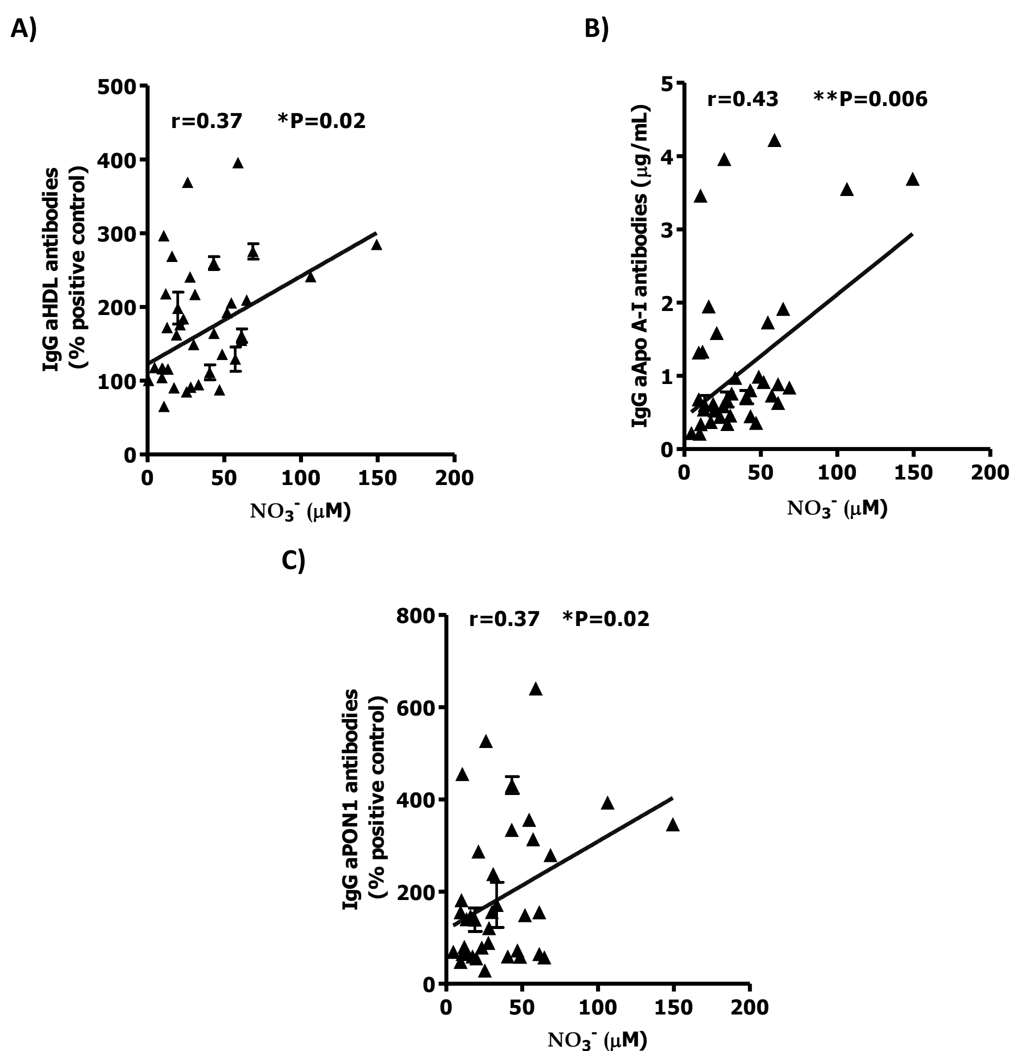


Figure 36. Correlation (Spearman's rank test) between NO_3^- levels and IgG aHDL (A), aApoA-I (B), aPON1 (C) antibodies titres in coronary artery disease (CAD) patients.

VCAM-1 levels showed a direct correlation with IgG aHDL (CAD: $p = 0.02$, IS: $p = 0.04$), aApo A-I (CAD and IS: $p = 0.01$) and aPON1 antibodies (CAD and IS: $p = 0.03$) in both groups of patients. In patients with CAD, the ICAM-1 levels showed a direct correlation with IgG aHDL ($p < 0.0001$), aApoA-I ($p = 0.0004$) and aPON1 antibodies ($p < 0.0001$). In patients with IS, ICAM-1 levels were only positively correlated with IgG aPON1 antibodies ($p = 0.02$). An inverse correlation was found between PON1 activity and VCAM-1 levels (CAD: $p = 0.01$ and IS: $p = 0.004$) in both groups of patients. ICAM-1 levels were negatively correlated with PON1 activity only in the IS group ($p = 0.009$).

In patients with CAD, NO_3^- levels showed a direct correlation with VCAM-1 ($p = 0.003$) and ICAM-1 ($p = 0.01$) while a positive correlation was observed between 3-NT levels and these adhesion molecules in IS patients (VCAM-1 and ICAM-1: $p = 0.01$).

Multivariate analysis entering the endothelial dysfunction markers as dependent variables and titters of IgG aHDL, aApoA-I and aPON1 antibodies as independent variables demonstrated that IgG aApoA-I titers independently predicted NO_3^- levels ($t = 2.92$, $p = 0.006$) while IgG aPON1 titers independently predicted ICAM-1 levels ($t = 3.97$, $p = 0.0001$) in patients with CAD.

3.2.1.4 Discussion

This study shows for the first time that antibodies towards HDL and two of its major components ApoA-I and PON1 are present in patients with IS and CAD. Previously, only aApoA-I antibodies had been identified in a cohort of patients with acute coronary syndrome and MI.[615-617] The lack of a positive titre of IgG aApoA-II and IgG aApoC-I across the two study groups rules against ApoA-II and ApoC-I as possible (auto)-antigens within the HDL complex, rather the strong correlation between IgG aHDL, aApoA-I and aPON1 titres suggests that ApoA-I and PON1 might be key targets. Moreover, the presence of IgG aHDL, aApoA-I and aPON1 antibodies in these subset of patients are associated with a decrease in PON1 activity and an increase in biomarkers of endothelial dysfunction (NO_3^-, 3-NT, VCAM-1 and ICAM-1), suggesting some biologic activity and potential pathogenicity.

It is widely accepted that elevated LDL and low HDL are independently related to the risk of vascular disease.[618] The anti-atherogenic properties of HDL are well established but neither the effect of aHDL antibodies on HDL's protective function, nor its relevance in patients without an auto-immune background is known. In this study the presence of IgG aApoA-I and aPON1 antibodies independently predicted decreases in PON1 activity and an increases in serum levels of endothelial dysfunction markers (NO_3^-, ICAM-1) indicating that the antioxidant/oxidant balance is tilted towards the latter with possible negative effects on the endothelial function.

The two NOS, the constitutive eNOS and the iNOS, convert L-arginine to NO• and citrulline at different concentrations according to substrate availability.[619] Of the NO• metabolites, it is generally accepted that in humans only NO_2^- reflects changes in eNOS activity and endothelial dysfunction whereas NO_3^- reflects mostly the "inflammatory" NO• from iNOS.[620-622] In keeping with these concepts serum NO_2^- was decreased in both patient groups whereas NO_3^- was markedly higher in CAD than IS. This may be a reflection of a more relevant role of inflammation in coronary diseases when compared to the brain-associated

injury. Moreover only CAD patients showed that IgG aApoA-I titers independently predicted increased NO_3^- levels, thus supporting an atherogenic role for IgG aApoA-I antibodies in CAD patients and highlight a different pattern of the two groups of patients regarding oxidation.

Physiological stimulation of the eNOS or iNOS enzyme enhances NO• release which then behaves either as a pathogenic mediator or as a cytotoxic molecule.[623,624] In the latter case NO• reacts with $O_2\bullet^-$ generating $ONOO^-$,[625] a strong oxidant that induces modifications in proteins, lipids and DNA by reacting with the tyrosine residues to form 3-NT which may in turn increase the immunogenicity of intracellular antigens, leading to a break in immune tolerance.[626] Nitrotyrosine (3-NT) has been widely used as a biomarker of protein damage induced by $ONOO^-$ and other reactive nitrogen species. In both groups of patients, 3-NT levels were increased when compared with healthy controls, however patients with CAD showed a positive relationship between 3-NT and IgG aHDL, aApoA-I antibodies, NO_3^-, whereas in IS patients 3-NT levels were positively association only with IgG aApoA-I antibodies and negative related with NO_2^- levels, again suggesting the possibility of different mechanisms in patients with cardio and cerebrovascular disease.

Levels of soluble adhesion molecules have been shown to correlate with various cardiovascular risk factors including low HDL-C levels.[608] Both groups of patients showed increased levels of ICAM-1 and VCAM-1 when compared with healthy subjects. Patients with CAD showed greater serum levels of ICAM-1 and VCAM-1 than IS patients though this difference was no obvious in previous surveys on cardio-vascular and cerebrovascular cohorts.[627-629] Although ICAM-1 has a low constitutive expression in a variety of cell types, VCAM-1 is not constitutively expressed, though up-regulation of the expression of both may be rapidly induced by pro-atherosclerotic conditions in animal models and in humans.[630-631] The clinical relevance of plasma adhesion molecules is not clear. In healthy individuals, an increase in ICAM-1 titres is a significant predictor of future CAD events whereas VCAM-1 is a significant predictor of future coronary events only in patients with pre-existing CAD.[632-636] In patients with IS a plasma concentration of VCAM-1 greater than 1350 ng/mL increases 4-fold the risk of new cerebrovascular events,[637] while iincrease of ICAM-1 is linked to a poor short term in acute stroke prognosis.[638]

In this study, VCAM-1 levels were positive associated with IgG aHDL, aApoA-I and aPON1 antibodies in both groups of patients, while ICAM-1 levels were positive associated with IgG aHDL, aApoA-I and aPON1 antibodies in CAD patients. In patients with IS, ICAM-1 levels were

only positive associated with IgG aPON1 and additionally IgG aPON1 titres independently predicted increased ICAM-1 levels in these group of patients.

This study reports the concurrent presence of IgG aHDL, aApoA-I and aPON1 antibodies in non autoimmune patients with atherosclerosis-associated clinical events together with markers of oxidation, nitrative stress and endothelial activation/damage. Outside the autoimmune disease context, only one group has shown a significant presence of aApoA-I antibodies in patients with acute coronary syndrome[615] and suggested that these antibodies increase atherosclerotic plaque vulnerability[617] and could be used as a prognostic marker in MI.[616] However they could do not found an association of the aApoA-I antibodies with other arterial or venous thromboembolic events, such as stroke or acute pulmonary embolism. Moreover, our study described for the first time the presence of aPON1 antibodies. The associations between antibodies titres and markers of oxidation, nitrative stress and endothelial activation/damage were found to be stronger in CAD patients than in IS patients, which can be explained by a greater inflammatory drive in CAD patients. Because of its cross sectional design, this study cannot explain whether the antibodies are causal to the clinical events, nevertheless the antibodies titres seems to be significant predictors of decreased HDL anti-atherogenic function and the associations found are consistent with a coherent pathogenic explanation.

3.2.2 Type 2 diabetes

Type 2 diabetes is primarily a metabolic disorder, with a major vascular involvement[31] and is a risk factor for the development of atherosclerosis. In fact the risk of developing atherosclerosis at an earlier age in patients with type 2 diabetes is three-to five fold greater that of nondiabetics after controlling for other risk factors.[32]

Recent findings indicate that HDL improve glucose metabolism by multiple mechanisms which include enhanced insulin secretion by β-cells as a result of improved cellular cholesterol homeostasis, protection of stress-induced apoptosis and islet inflammation[303-305] and may be a causative role in the development of insulin resistance.[303]

Infusions of rHDL in patients with type 2 diabetes reduce plasma glucose, increase insulin secretion, improve the HOMA index when compared with placebo[306] and can inhibit

the cellular expression of VCAM-1 and ICAM-1 on stimulated human coronary endothelial cells.[639]

Dysfunctional HDL has been identified in patients with type 2 diabetes: these show an impaired HDL-mediated RCT as well as defaults in the anti-oxidative, anti-inflammatory and endothelial-protective features of HDL.[640] Dysfunctional HDL can be due to changes in its multiple components, but is most frequently associated with ApoA-I glycation.[327]

We confirmed the presence of antibodies towards HDL components outside the context of autoimmune diseases, in patients with CAD and IS. Due to the potential role of HDL in the pathogenesis of type 2 diabetes, together with the known increased atherosclerotic risk of these patients, we looked for the presence of aHDL antibodies also in this disease.

3.2.2.1 Aim

This study was undertaken to determine the presence of antibodies directed against different components of HDL in patients with type 2 diabetes and establish a possible relationship between these antibodies and the anti-oxidant and anti-inflammatory properties of HDL.

3.2.2.2 Patients and methods

Patients

Seventy four consecutive patients with type 2 diabetes, followed at the Internal Medicine outpatients clinic of Curry Cabral Hospital from June 2010 to June 2011, where invited to participate in this study. Seventeen declined participation and 57 accepted. Inclusion criteria were routine follow up appointment, diagnosis of type 2 diabetes according to American Diabetes Association guidelines 2008[641]: fasting plasma glucose (FPG) ≥ 126 mg/dL (7.0 mmol/L) or two-hour plasma glucose ≥200 mg/dl (11.1 mmol/L) during an glucose tolerance test (OGTT) or classic symptoms of hyperglycaemia such as polyuria, polydipsia, and unexplained weight loss. Exclusion criteria included diabetes mellitus other than type 2; disease, renal or hepatic (serum creatinine < 1.2 mg/dL and ALT/AST < 2x reference value) dysfunction, infection, neoplastic disease, history of alcoholism or drug abuse, history of mental illness

Of the initial cohort, 7 patients with type 2 diabetes were excluded to match with the control group for age (the oldest patients were excluded), fifty patients were definitely enrolled in the study.

Healthy subjects used as controls were recruited during the same period of time from healthy blood donors and family and friends of hospital staff.

Demographic data of patients and controls, medical history (including current medication) and other clinical data are summarized in Table 11.

All subjects signed consent forms approved by the ethics committees of the Hospital and the study was carried out according to the revised declaration of Helsinki.

Blood samples were collected after a 12h fast and were centrifuged at 3000 xg at 4ºC for 10 min to obtain serum. Serum from patients and controls were kept at –80°C until assayed.

Methods

Serum IgG aHDL, IgG aApoA-I, IgG aPON1, IgG aApoA-II and aApoC-I antibodies titers and levels of soluble VCAM-1, ICAM-1 and 3-NT were measured by ELISAs. PON1 activity and nitric oxide metabolites (NO_2^- and NO_3^-) levels were tested as described in methods. Lipid profile (total cholesterol, HDL-C, LDL-C and TG) were determined by standard enzymatic techniques in the hospital when samples were collected. ApoA-I levels were determined by immunoturbidimetry as described in methods chapter.

3.2.2.3 Results

Characteristics of the study subjects

Type 2 diabetic patients had a greater body mass index than controls and were more likely to present with co-morbidities such hypertension (50%) and dyslipidemia (22%) and to be on medication (Table 11).

FPG levels (180.0 ± 95.4 mg/dL) and HbA1c (9.1 ± 4.9 %) have been used as the indices of glycaemia as they were measured in all subjects and they were significantly increased in patient with diabetes, consistent with uncontrolled diabetes (Table 11).

HDL-C and ApoA-I were significantly lower in patients with diabetes when compared with controls, but other lipid parameters were not significantly different across subject groups (Table 11). In patients with diabetes, HDL-C levels were inversely correlated with FPG levels ($r = -0.41$, $p = 0.004$) and both HDL-C and ApoA-I levels were negatively associated with HbA1c ($r = -0.42$, $p = 0.003$).

Table 11. Demographic characteristics and clinical and serological data of healthy controls (CTRL) and patients with type 2 diabetes.

	CTRL (n = 50)	**Type 2 diabetes (n = 50)**	**P value**
Age (years)	67 ± 12	71 ± 10	0.06
Sex: female/male (n)	24 / 26	21 / 29	0.68
BMI (Kg/m^2)	28.6 ± 4.0	32.1 ± 5.5	<0.0001
Medical History, N (%)			
Hypertension	5(10)	25 (50)	<0.0001
Dyslipidemia	2(4)	11 (22)	0.01
Medication, N (%)			
Sulfonylureias	0	11 (22)	0.0005
Biguanides	0	16 (32)	<0.0001
Insulins	0	5 (10)	0.05
Antihypertensives drugs	5(10)	17 (34)	0.007
Antidyslipidemic drugs	2(4)	9 (18)	0.05
Serological data			
FPG (mg/dL)	89.5 ± 10.8	180.0 ± 95.4	<0.0001
HbA1c (%)	5.1 ± 1.3	9.1 ± 4.9	<0.0001
HDL-C (mg/dL)	55.0 ± 22.7	37.4 ± 12.3	<0.0001
LDL-C (mg/dL)	89.2 ± 32.7	90.0 ± 35.3	0.76
Total cholesterol (mg/dL)	163.3 ± 53.3	149.3 ± 45.2.	0.07
TG (mg/dL)	113.7 ± 51.8	135.7 ± 55.9	0.14
ApoA-I (mg/dL)	147.6 ± 26.8	121.7 ± 27.2	<0.0001

Abbreviations: Body mass index (BMI); Fasting plasma glucose (FPG); Glycosylated hemoglobin (HbA1c); Triglycerides (TG); Apolipoprotein A-I (ApoA-I). Data are presented as mean ± SD. P value represents the statistical comparison between the two groups. Mann Whitney test.

IgG aHDL, aApoA-I and aPON1 antibodies in patients with type 2 diabetes

Mean titres of IgG aHDL, aApoA-I and aPON1 antibodies were higher in patients with type 2 diabetes than healthy controls (Figure 37 A-C; $p < 0.0001$, $p = 0.0005$, $p < 0.0001$, respectively). No other differences were observed for the remaining antibodies (IgG aApoA-II, aApoC-I) tested (Table 12).

The prevalence of positive titres of IgG aHDL antibodies were 66% in patients with type 2 diabetes and 26% in healthy controls, whilst for aApo A-I antibodies were 48% and 10% for patients type 2 diabetes and healthy controls, respectively. The prevalence of positive titres of IgG aPON1 antibodies in patients with type 2 diabetes were 64% and in healthy controls 8%.

IgG aHDL directly correlates with the IgG aApoA-I titers ($r = 0.56$, $p < 0.0001$) and IgG aPON1 titers ($r = 0.52$, $p < 0.0001$) in patients with type 2 diabetes.

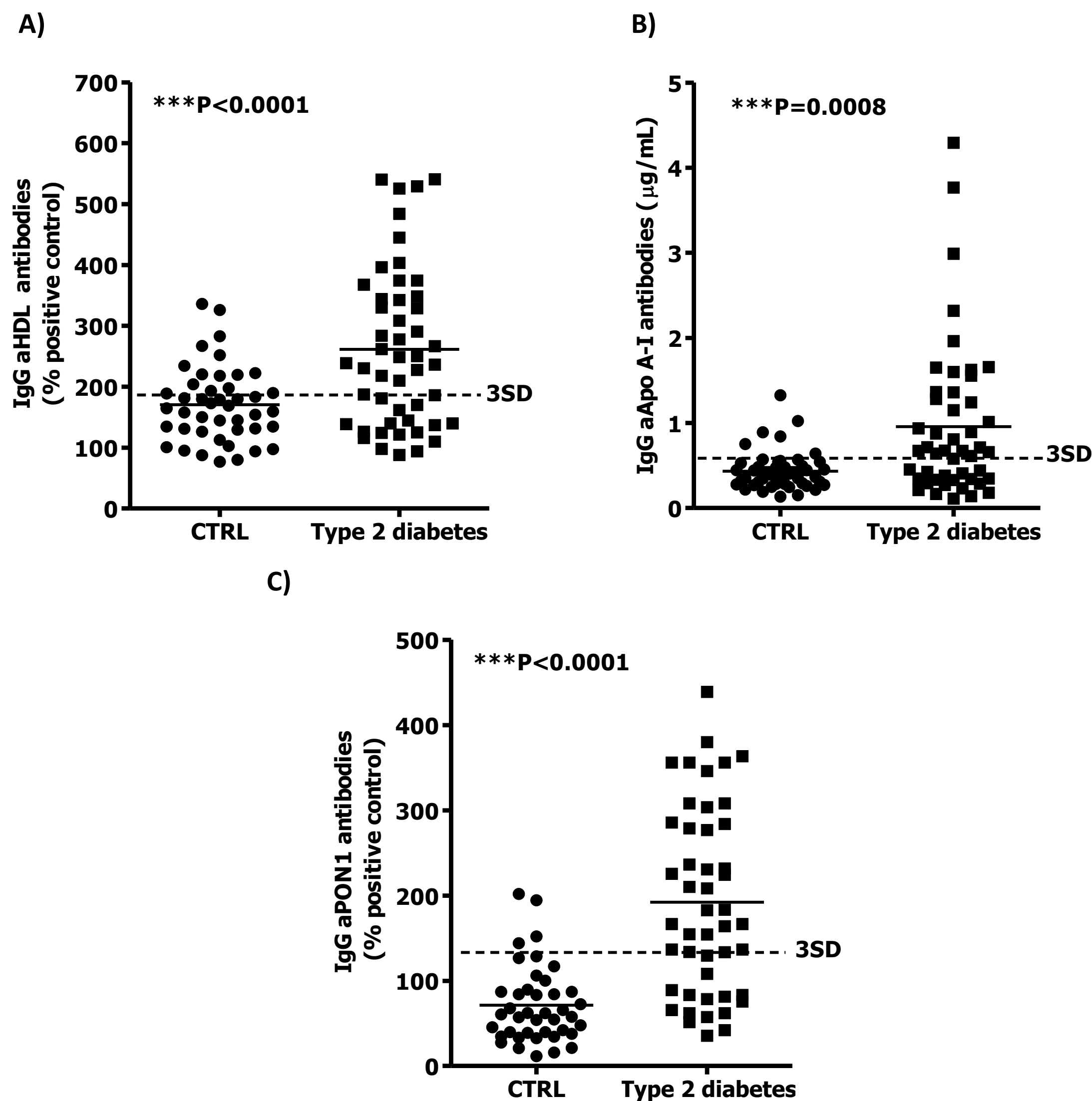


Figure 37. Levels of IgG aHDL (A), aApoA-I (B) and aPON1 (C) antibodies in healthy controls (CTRL) and in patients with type 2 diabetes. Bars show the means. Mann Whitney test.

Table 12. Antibodies titres measured in healthy controls (CTRL) and in patients with type 2 diabetes.

	CTRL	Type 2 diabetes	P value
IgG aHDL (% p. control)	170.6 ± 61.56	261.5 ± 130.1	<0.0001
IgM aHDL (% p. control)	35.5 ± 24.6	30.14 ± 22.0	0.48
IgG aApoA-I (µg/mL)	0.43 ± 0.23	0.96 ± 0.89	0.0008
IgG aApoA-II (% p. control)	126.8 ± 78.33	153.2 ± 75.5	0.21
IgG aApo C-I (% p. control)	289.3 ± 158.2	344.1 ± 202.6	0.28
IgG aPON1 (% p. control)	71.3 ± 45.5	192.0 ± 108.4	<0.0001

Abbreviations: aHDL: anti-high density lipoprotein antibodies; aApo A-I: anti-apolipoprotein A-I antibodies; aApo A-II: anti-apolipoprotein A-II antibodies aApo C-I: anti-apolipoprotein C-I antibodies; aPON1: anti-paraoxonase 1 antibodies. Mann Whitney test.

Patient's fasting glucose, glycosylated haemoglobin levees and lipid profile association with titres of antibodies

FPG levels of patients with type 2 diabetes were positively correlated with the titres of IgG aHDL ($r = 0.49$, $p < 0.0001$), aApoA-I ($r = 0.36$, $p = 0.01$) and aPON1 ($r = 0.78$, $p < 0.0001$) antibodies. Levels of HbA1c were also positively correlated with IgG aHDL ($p < 0.0001$), aApoA-I ($p = 0.01$) and aPON1 ($p < 0.0001$) antibodies (Figure 38 A-C).

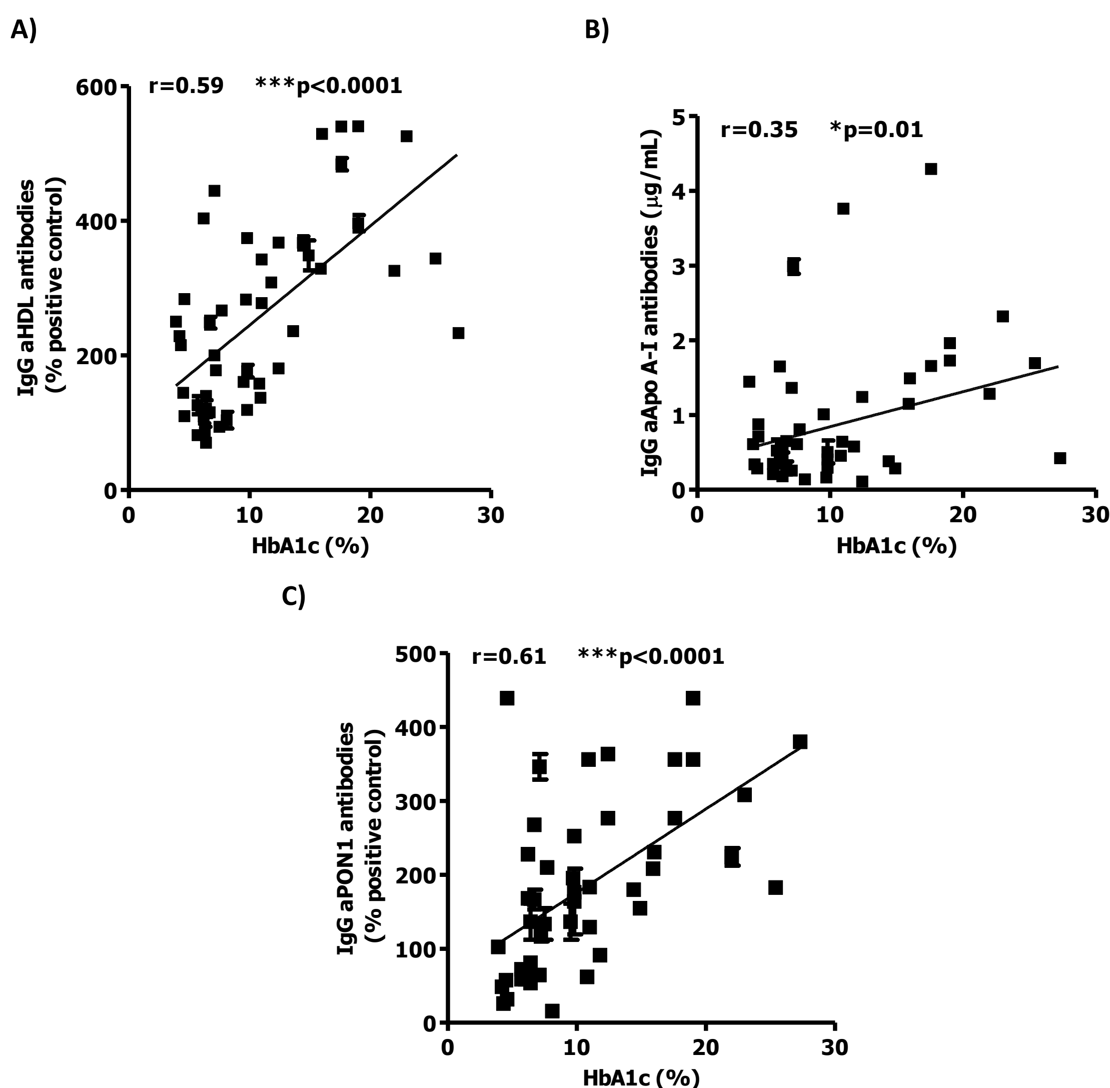


Figure 38. Correlation (Spearman's rank test) between glycosylated hemoglobin (HbA1c %) and IgG HDL (A), aApoA-I (B) and aPON1 (C) antibodies in patients with type 2 diabetes.

HDL-C levels were negatively correlated with the titres of IgG aHDL ($r = -0.47$, $p = 0.0006$), aApoA-I ($r = -0.38$, $p = 0.008$) and aPON1 ($r = -0.45$, $p = 0.001$) antibodies. Whilst ApoA-I levels were only negatively correlated with the presence of IgG aPON1 antibodies ($r = -0.40$, $p = 0.004$).

In a regression model including FGP levels or HbA1c as the dependent variables and age, gender, HDL-C, LDL-C, total cholesterol, TG, ApoA-I levels, PON1 activity, IgG aHDL, aApoA-I and aPON1 antibodies titres as independent variables, IgG aPON1 antibodies independently predicted FGP levels ($t = 4.52$, $p = 0.0001$), whilst IgG aHDL antibodies independently predicted increased HbA1c ($t = 3.49$, $p = 0.001$).

Moreover a regression model including HDL-C as the dependent variable and age, gender, LDL-C, total cholesterol, TG, ApoA-I levels, PON1 activity, IgG aHDL, aApoA-I and aPON1 antibodies titres as independent variables, Apo A-I levels were independently predicted HDL-C levels ($t = 5.53$, $p = 0.0001$), whilst IgG aApoA-I antibodies negatively predicted HDL-C levels ($t = -3.05$, $p = 0.006$).

PON1 activity and association with higher titres of antibodies

PON1 activity was lower in patients when compared to healthy controls ($p<0.0001$, Table 13) and correlated in a negative fashion with the titres of IgG aHDL ($p = 0.009$), aApoA-I ($p = 0.01$) and aPON1 ($p = 0.005$) antibodies (Figure 39 A-C). Moreover PON1 activity directly correlated with ApoA-I levels ($r = 0.32$, $p = 0.04$) but was lower when associated with FGP levels ($r = -0.30$, $p = 0.04$).

Table 13. Biological variables (oxidation and inflammation markers) measured in healthy controls (CTRL) and in patients with type 2 diabetes.

	CTRL	Type 2 diabetes	P value
PON1 activity (U/L)	274.0 ± 78.9	174.0 ± 92.6	<0.0001
NO_2^- (μM)	13.7 ± 8.7	15.5 ± 10.9	0.67
NO_3^- (μM)	25.5 ± 17.0	42.3 ± 31.5	0.008
3-NT (nM)	16.3 ± 6.0	20.7 ± 11.0	0.25
VCAM-1 (ng/mL)	723.6 ± 342.4	1307.0 ± 635.1	<0.0001
ICAM-1 (ng/mL)	286.7 ± 90.9	452.5 ± 234.2	0.0002

Abbreviations: PON1: paraoxonase 1; NO_2^-: nitrite; $NO3^-$: nitrate; 3-NT: 3-nitrotyrosine; VCAM-1: vascular cell adhesion molecule; ICAM-1: intracellular adhesion molecule.

In this cohort of patients with type 2 diabetes, IgG aPON1 antibodies titres independently predicted PON1 activity ($t = -3.68$, $p = 0.001$) in a negative fashion in a multivariate analysis.

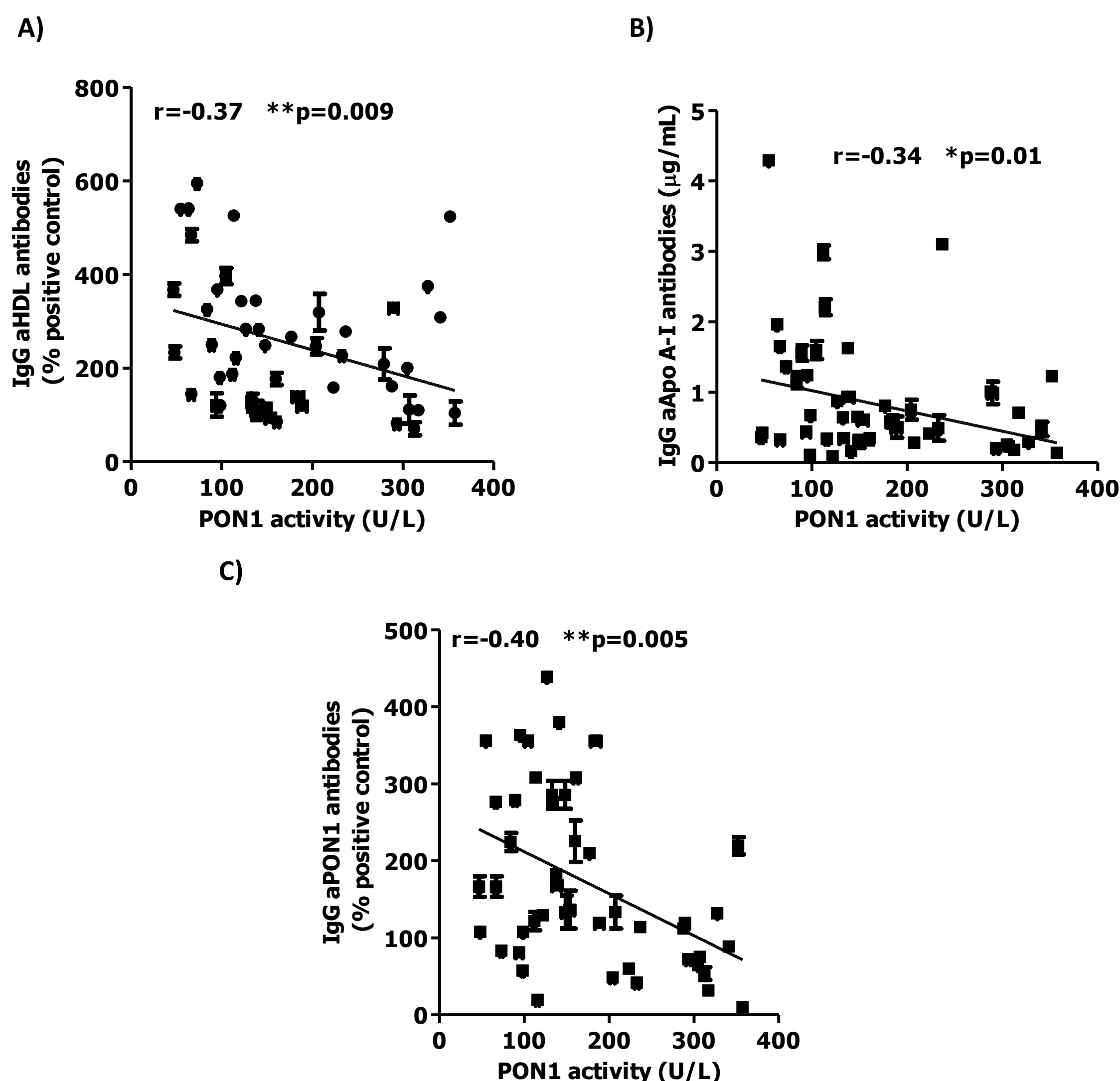


Figure 39. Correlation (Spearman's rank test) between PON1 activity (U/L) and IgG HDL (A), aApoA-I (B) and aPON1 (C) antibodies in patients with type 2 diabetes.

Endothelial dysfunction parameters and association with higher titres of antibodies

There were no differences between mean NO_2^- levels in healthy control and patients with type 2 diabetes, but NO_3^- levels were increased when compared with healthy controls ($p = 0.008$) (Table 13). No association were observed between NO_3^- and the antibodies titers in type 2 diabetes. Also there were no differences regarding 3-NT levels between healthy control and patients with type 2 diabetes.

VCAM-1and ICAM-1 levels were increased in patients with type 2 diabetes, when compared to healthy controls, ($p < 0.0001$, $p = 0.0002$, respectively) (Table 13). VCAM-1 levels showed a direct correlation with IgG aApoA-I ($p = 0.0001$) and aPON1 ($p < 0.0001$) antibodies (Figure 40 A-B).

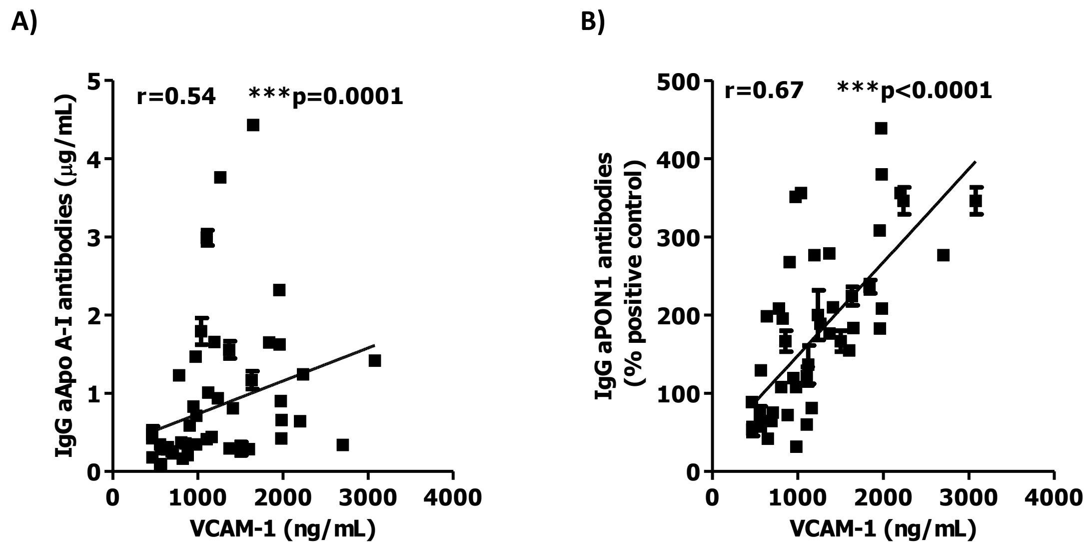


Figure 40. Correlation (Spearman's rank test) between VCAM-1 (ng/mL) levels and IgG aApoA-I (A) and aPON1 (B) antibodies in patients with type 2 diabetes.

VCAM-1 levels were inversely correlated with HDL-C (r = -0.46, p = 0.001), ApoA-I levels (r = -0.46, p = 0.002) and PON1 activity (r = -0.39, p = 0.009). ICAM-1 levels were directly correlated with IgG aHDL (r = 0.35, p = 0.02), aApoA-I, aPON1 antibodies(r = 0.43, p = 0.006) and with FGP levels (r = 0.39, p = 0.01). An inverse correlation was found between ICAM-1 and ApoA-I (r = -0.52, p = 0.0008) levels and with PON1 activity (r = -0.36, p = 0.02) in patients with type 2 diabetes.

In a regression model including VCAM-1 as the dependent variable and HDL-C, LDL-C, total cholesterol, TG, ApoA-I, FGP levels, HbA1c, PON1 activity, IgG aHDL, aApoA-I and aPON1 antibodies titres as independent variables, IgG aPON1 antibodies independently predicted VCAM-1 levels (t = 3.89, p = 0.001) whereas HDL-C negatively predicted VCAM-1 levels (t = -2.41, p = 0.02). Using the same regression model but with ICAM-1 as a dependent variable aApoA-I levels negatively predicted ICAM-1 (t = -2.51, p = 0.02).

3.2.2.4 Discussion

This study shows for the first time that antibodies towards HDL and two of its major components ApoA-I and PON1 are presented in patients with type 2 diabetes. The absence of aApoA-II and aApoC-I antibodies suggests that this is a specific immune response towards HDL, indeed the strong correlation between IgG aHDL, aApoA-I and aPON1 suggests that ApoA-I and PON1 might be key targets.

The relationship between HDL-C levels and glucose metabolism has been widely studied.[642,643] In our cohort the inverse relationship between FPG levels or HbA1c % and

HDL-C and ApoA-I levels were confirmed. Moreover titres of IgG aHDL antibodies independently predicted increased HbA1c while IgG aPON1 antibodies were independently associated with higher FGP levels and consequently with poor glycaemia control suggesting that these antibodies may interfere with the protective effect of HDL on glucose metabolism.

Furthermore, the presence of IgG aHDL, aApoA-I and aPON1 antibodies in these subset of patients was also associated with a decrease of HDL-C and ApoA-I levels and PON1 activity and an increase of adhesion molecules (VCAM-1 and ICAM-1).

Chronic hyperglycaemia leads to generation of advanced gyration end products (AGEs), products from the nonenzymatic glycation of reducing sugars with macromolecules which are directly involved in endothelial dysfunction in these patients. The binding of AGEs to its receptor (RAGEs) activates intracellular signalling processes triggering AGE-mediated pro-inflammatory effects.[644,645] AGE modification is known to occur not only with hemoglobin (HbA1c), but also with various proteins, thus affecting their functions. In fact, it has been reported that nonenzymatic glycation of HDL, ApoA-I and PON1 *in vitro* affect their anti-atherogenic properties, in particularly impairs HDL anti-inflammatory and antioxidant functions.[329,646,647] Such modifications in proteins and lipids may increase the immunogenicity of intracellular antigens, leading to a break in immune tolerance.

These findings suggest that antibodies towards HDL are associated with an increased risk of oxidative stress in these patients and therefore contributing to the accelerated atherogenesis present in type 2 diabetes.

As mentioned previously NO_2^- levels seem to reflect changes in eNOS activity and endothelial dysfunction whilst NO_3^- reflects mainly the “inflammatory” NO• from iNOS.[620-622] It is also known that iNOS, which mediates the pro-apoptotic effects of glucose is down-regulated by HDL.[302]

In this cohort, patients with type 2 diabetes showed increased levels of VCAM-1 and ICAM-1 when compared with healthy subjects and a positive association with IgG aHDL, aApoA-I and aPON1 antibodies. In particularly IgG aPON1 antibodies which independently predict increased VCAM-1 levels, suggesting that this antibodies may interfere with the anti-inflammatory properties of HDL.

This study describes the concurrent presence of IgG aHDL, aApoA-I and aPON1 antibodies in patients with type 2 diabetes and its association with markers of oxidation, nitrative stress and endothelial activation/damage

These results add to the mounting evidence of greater than expected humoral auto-immunity in non autoimmune patients with atherosclerotic related clinical events. Prospective studies are now required to confirm whether these antibodies are indeed a direct cause of the identified immune changes.

4. BIOLOGIC ACTIVITY AND PATHOGENIC POTENTIAL OF ANTIBODIES TOWARDS THE HDL COMPLEX

4.1 Introduction

In the previous chapter it was proposed that a humoral response against the HDL complex could account for a quantitative and/or qualitative fault of this lipoprotein.

aHDL antibodies and more particularly aApoA-I (the main apolipoprotein contained in HDL) and aPON1 (the main antioxidant enzyme in HDL) antibodies are present in patients with autoimmune diseases but also in patients with atherosclerosis-associated clinical events outside the context of autoimmune diseases. Furthermore an association was found between the titres of these antibodies and several biomarkers of oxidation and endothelial dysfunction. However, these findings were reported in cross-sectional clinical studies and therefore a causal relationship could not be inferred. Prospective studies would be needed to determine the pathogenic effect of these antibodies. Nevertheless, the pathogenicity of these antibodies can be assessed in *in vitro* systems.

The knowledge that aHDL antibodies purified from patients might interfere with defined physiologic mechanisms could provide strong evidence for their pathogenic potential and reject their presence as simple bystanders. So far, antibodies against the HDL complex have not been previously isolated and therefore proper experiments regarding its biological activity are needed.

4.2 Aim

The goal of this study is to demonstrate the biologic activity and consequent pathogenic capacity of the antibodies towards the HDL complex by isolating them from patient's serum and then studying there *in vitro* effects on the anti-oxidant and anti-inflammatory properties of HDL.

4.3 Patients and Methods

Patients

Patients with SLE and CAD and IS from the cohorts previously studied who had aHDL antibodies were selected and the respective serum (already stored) was used.

Methods

Antibodies from patients with SLE were isolated using a 96-well microtitre plate as solid phase, following the protocol described in methods. The aHDL antibodies from patients with

CAD and IS were isolated by immunoaffinity chromatography using a HiTrap NHS- activated HP column following the protocol explained in methods.

Inhibition of PON1 activity by aHDL and aApoA-I antibodies isolated from SLE patients was performed as described in previously in 2.3.1.1, whilst inhibition of PON1 activity by aHDL antibodies isolated from a pool of patients with CAD and IS was performed as described in section 2.3.1.2.

The effect of the aHDL antibodies isolated from a pool of patients with CAD and IS on expression of VCAM-1 and in VEGF levels in HUVECs was studied as described in section 2.3.2 and 2.1.1.8, respectively.

The results and discussion of each set of experiment will be presented in separated sections

4.4 Isolation of aHDL and aApoA-I antibodies from patients with SLE and in vitro inhibition of PON1 activity

4.4.1 Results

The aHDL and aApo A-I antibodies isolated from patients with SLE and incubated with human HDL significantly reduced PON1 activity up to a maximum of 70.2% and 78.4% respectively, when compared to irrelevant human IgG (Figure 41 A and B). The inhibition of PON1 activity was dose-dependent in relation to the concentration of the antibody incubated with different known concentrations of HDL.

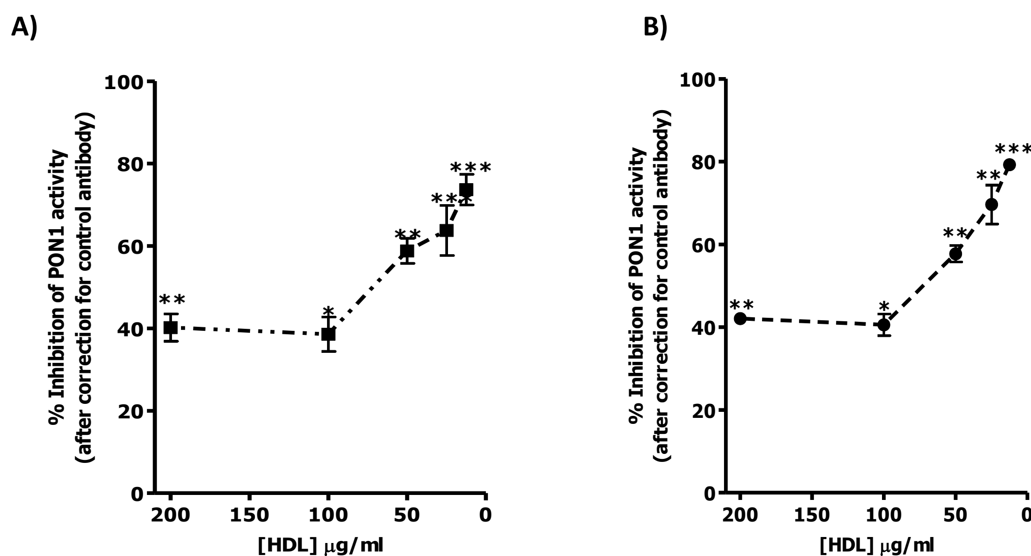


Figure 41. *In vitro* inhibition of paraoxonase 1 (PON1) activity by aHDL (A) and aApoA-I (B) antibodies isolated from serum patients with systemic lupus erythematosus (SLE). Human IgG was used as control and correspond to 0% of effect. ***$p<0.001$; **$p<0.01$; *$p<0.05$.

4.4.2 Discussion

The aHDL and aApoA-I antibodies isolated from SLE patients at a constant concentration directly inhibited the activity of PON1 in a dose dependent fashion. This complementary set of experiments demonstrates that aHDL and aApoA-I interfere with HDL disrupting the normal activity of PON1. The negative correlations found in the SLE cohort analysis in the previous chapter between the antibodies titres and the PON1 activity were confirmed by this *in vitro* assay.

The purification protocol allowed only a small recovery volume after isolation and it was not possible to determine the concentration of IgG aHDL and aApo A-I antibodies, therefore the dose dependency inhibition of PON1 was evaluated by incubating the fixed antibody concentration with decreasing concentrations of antigen (HDL).

4.5 Immunoaffinity chromatography purification of aHDL antibodies from patients with IS and CAD

4.5.1 Results

The profile of the HDL coupling to the HiTrap NHS activated HP column at 280mn was represented in Figure 42.

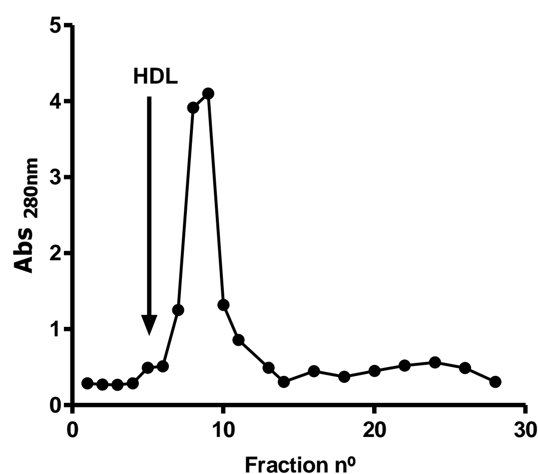


Figure 42. Representative profile of the HDL coupling to the HiTrap NHS activated HP column at 280nm.

Figure 43 shows the typical profile of serum patients; composed by a bulk of unretained proteins that passed directly through the column (fractions from 14-17), a second peak of retained proteins that were eluted when the buffer was changed to 0.1 M Glycine pH 2.5 (fractions from 30-32) and a third peak appeared after washing the column with 20 mM Tris pH 8.8 (fractions from 39-41).

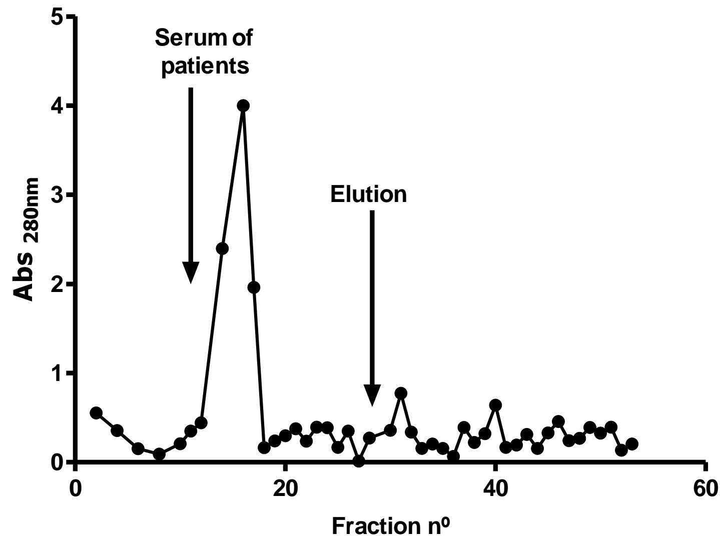
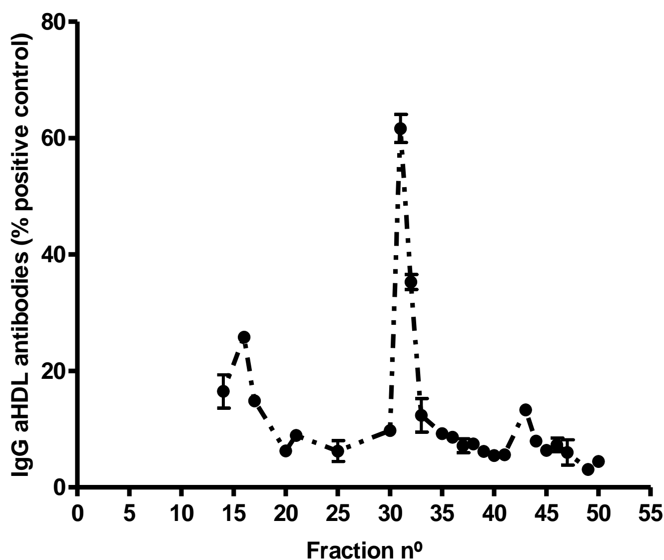


Figure 43. Representative elution profile of a pool of serum patients with atherosclerosis-associated clinical events: coronary artery disease (CAD) and ischemic stroke (IS) and applied to a HDL-HiTrap NHS activated HP column at 280nm. Proteins which bound to the column were eluted with 0.1 M Glycine pH 2.5.

All the collected fractions were tested for the presence of IgG aHDL antibodies (Figure 44 A) and they were only identified on fractions 31 and 32.

The IgG aHDL antibodies isolated retained the ability to bind to human HDL (Figure 44 B) and the recovery rate in the column was greater than 90%. The fractions 31 and 32 were pooled and concentrated to test the biologic activity of the isolated aHDL antibodies.

A)



B)

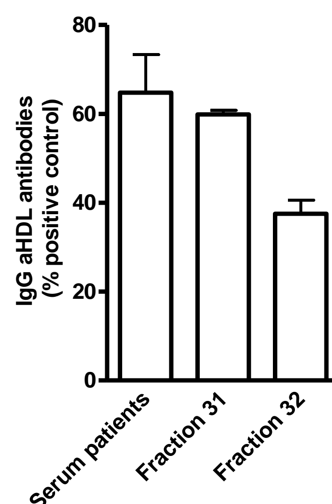


Figure 44. Levels of IgG aHDL antibodies in the fractions collected from the HDL- HiTrap NHS activated HP column after patients serum was applied (A). Capacity of recovery of aHDL antibodies from fractions 31 and 32 in relation to the original patients samples applied to the column (B). Bars show means.

4.5.2 Discussion

The main new finding of this experiment was the development of a methodology for the isolation of aHDL antibodies from serum of patients, using immunoaffinity chromatography with a HDL - HiTrap NHS activated HP column.

Immunoaffinity chromatography is a general method for the isolation of antibodies found in biologic fluids. The use of a pre-packed column has several advantages over the first protocol (Antibodies isolated in a 96-well microtitre as solid phase) since it allows the purification of larger amounts and more concentrate antibodies at the same time maintaining their specificity and preserving their biological activity. Furthermore, columns can be re-used if stored in adequate, though the matrix adsorbed on the column has a limited lifetime, and the ligand coupled to the matrix can suffer oxidation (or other biologic modification), which may interfere with the isolated antibody.

This protocol was developed to purify aHDL antibodies from serum of patients with atherosclerotic clinical events (IS and CAD). However, it is a method that is generally applicable for the purification of antibodies towards different HDL components (ApoA-I and PON1) and also in other pathologies such as SLE.

4.6 In vitro inhibition of PON1 activity by aHDL antibodies

4.6.1 Results

aHDL antibodies isolated from patients with CAD and IS inhibited PON1 activity in a dose-dependent fashion from 7% to 52% (Figure 45). The non-specific human IgG used as control did not affect PON1 activity when compared to basal conditions (0% effect).

4.6.2 Discussion

This experiment showed that aHDL antibodies purified from CAD and IS patients directly inhibit PON1 activity, supporting the previous clinical studies where aHDL was associated with a pro-oxidant effect and a consequent increase in oxidative stress via a disruption of the normal activity of PON1 (sub-chapter 3.2.1).

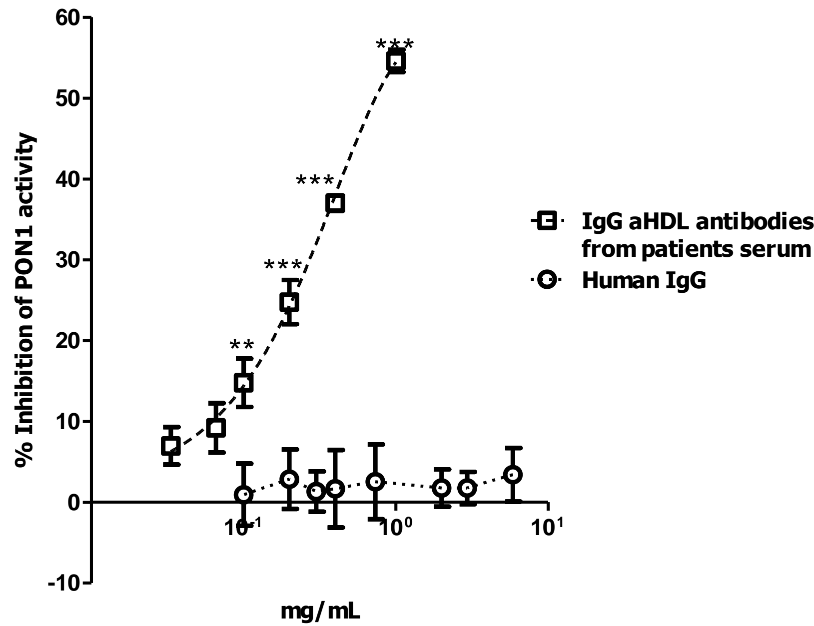


Figure 45. *In vitro* inhibition of paraoxonase 1 (PON1) activity by IgG aHDL antibodies isolated from serum of patients with atherosclerosis-associated clinical events (CAD and IS). Human IgG was used as control and 0% effect is the PON1 activity at basal conditions (HDL 100μg/mL).

4.7 *In vitro* exposure of human umbilical vein endothelial cells (HUVECs) to aHDL antibodies

4.7.1 Results

Unstimulated HUVECs do not express VCAM-1 (incubation of HUVECs with HDL induced a 26.4 ± 11.3 % increase in the expression of VCAM-1 compared to baseline which might be likely attributed to an artefactual oxidation of HDL during isolation). VCAM-1 expression can be induced by stimulation with TNF-α, in this experiment there was an increase of 213.7 ± 55.4 % of VCAM-1 expression in comparison to baseline. The pro-inflammatory effect of the cytokine was prevented by pre-incubation of HUVECs with HDL which led to a marked reduction of 160% in the subsequent expression of VCAM-1 (p=0.0004). In contrast, the addition of the aHDL antibodies isolated from patients to the pre-incubation solution abrogated the inhibitory effect of HDL on VCAM-1 expression by more than 80%, when compared with the non-specific human IgG (Figure 46). The presence of aHDL antibodies alone or with TNF-α (without HDL) did not modified the expression of VCAM-1 on HUVECs. Additionally the pre-incubation of HUVECs with human monoclonal aApo A-I antibody prevented the effect of the pro-inflammatory cytokine by only 65% in comparison to non-specific human IgG.

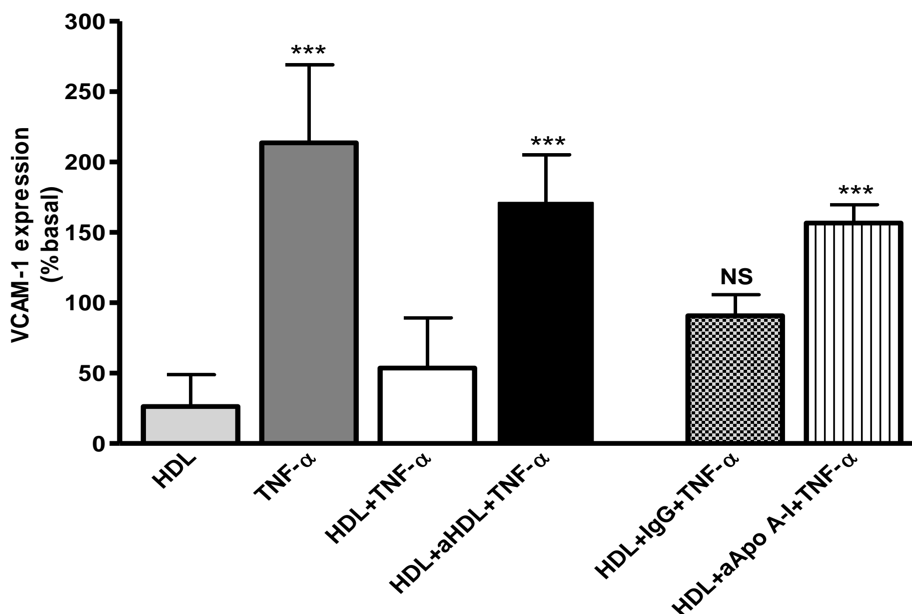


Figure 46. Effect of aHDL antibodies isolated from patient's serum on the expression of VCAM-1 in HUVECs exposed to TNF-α. Confluent cultured HUVECs were incubated in basaline conditions (0% effect), with human HDL (1.6mg/mL), TNF-α (10ng/mL), HDL + TNF-α alone or with aHDL antibodies (50µg/mL) isolated from serum of patients with atherosclerosis-associated clinical events or a non-specif human IgG (50µg/mL) or human monoclonal aApo A-I antibody (30µg/mL). Bars show the means ± SD. Differences between means were evaluated using an Bonferroni's Multiple Comparasion test. ***P<0.0001 with reference to HDL + TNF-α; NS not significant.

HDL induced a 157% increase in the levels of VEGF produced by HUVECs in comparison with baseline. aHDL antibodies isolated from patients decreased HDL-associated VEGF levels by 65%, whilst the incubation with human monoclonal aApo A-I antibody did not change the VEGF levels (Figure 47).

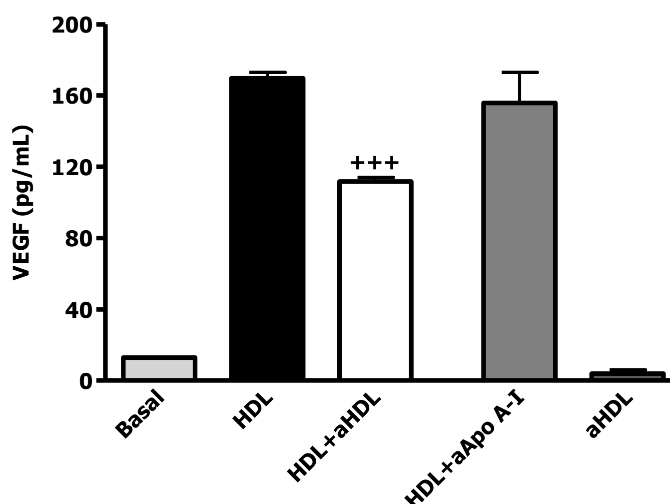


Figure 47. Effect of aHDL antibodies isolated from patients serum on VEGF levels produced by HUVECs. Cells were exposed to HDL alone or with aHDL antibodies (50µg/mL) or human monoclonal aApo A-I antibody (30µg/mL). Bars show the means ± SD. Differences between means were evaluated using an Bonferroni's Multiple Comparasion test. $^{+++}$P<0.0001 with reference to HDL.

4.7.2 Discussion

The interaction of leucocytes with the vascular endothelium is pivotal to the inflammatory process, and is mediated, amongst others, by adhesion molecules such as VCAM-1. They participate in the early events of atherogenesis by promoting monocyte adhesion to the endothelium and their subsequent migration through the arterial intima. Levels of soluble adhesion molecules have been shown to correlate with various cardiovascular risk factors including low HDL-C levels.[608] In fact, the ability of HDL as well as some of its constituents, to inhibit cytokine-induced upregulation of cell surface adhesion molecules expression has been consistently demonstrated *in vitro* with native HDL[213,648] and reconstituted HDL.[234,235] This inhibition has been documented in endothelial cells activated with both TNF-α and IL-1.

However the mechanism by which HDL interacts with the cells to inhibit adhesion molecules expression is not fully understood. Some studies suggest that HDL blocks the sphingosine kinase (SphK) pathway, an enzyme catalysing a key step in endothelial cell activation by TNF-α.[649]

In the present study we confirmed the anti-inflammatory property of HDL by inhibiting the TNF-α induced expression of VCAM-1 in HUVECs. Furthermore, by adding aHDL antibodies isolated from patients to the pre-incubation solution, the inhibitory effect of HDL on VCAM-1 expression was blocked, confirming the biologic activity of these antibodies.

In previous studies (Chapter 3) adhesion molecules levels were directly associated with the antibody titres both in SLE patients and in non-autoimmune patients. Although, this experiment tested only aHDL antibodies isolated from patients with IS and CAD the inhibitory effect of the monoclonal human aApo A-I antibody suggests that aApo A-I antibodies isolated from autoimmune or no-autoimmune patients might also exert an inhibitory effect on VCAM-1 expression. This pro-inflammatory effect of the aApo A-I antibodies is not surprising due to the known anti-inflammatory properties of Apo-I.[242,239] In fact, the present results suggest that HDL may exert its anti-inflammatory properties via an ApoA-I dependent mechanism.

Angiogenesis is a critical component of several human diseases, including cancer, auto-immune diseases, ocular neovascularizing disorders and CVD. VEGF plays a central role in this process and in most of these diseases increased levels of VEGF is likely to contribute to disease progression. However, in ischemic heart and peripheral vascular disease the problem

is one of vascular insufficiency. In fact, autocrine VEGF is required for the homeostasis of blood vessels in the adult.[650] As matter of fact in animal models non-invasive injection of pro-angiogenic compounds such as VEGF has shown promising results in regenerating cardiac microvasculature, however these results have failed to translate into successful clinical trials, although the motive still unclear.[651]

In the present study we also confirmed that HDL-induces angiogenesis[652] by increasing VEGF levels in HUVECs. The addiction of aHDL antibodies isolated from patients to the pre-incubation solution abrogated the positive effect on VEGF levels. Unlike what was observed on VCAM-1 expression, human aApo A-I antibody did not exert the same effect of the aHDL antibodies isolated from patients with IS and CAD. This result suggest that HDL-induced angiogenesis by a mechanism independent of ApoA-I and can be due to other components of the HDL complex.

5. PHARMACOLOGIC MANIPULATION OF HDL-C AND aHDL ANTIBODIES TITRES

This chapter will deal with three blind / randomized clinical trials with different anti-dyslipidemic drugs in which the primary endpoint was not the evaluation of the effect of the drug on the humoral response towards HDL, nevertheless this effect was evaluated in a subsequent analysis. None of these studies protocol was written / developed by myself however for a better understanding of the study they will be described. All the measurements of biochemical and immunological variables presented here in were performed by me and included in this thesis with the respective authorization of clinicians that developed these clinical trials.

5.1 Exploratory, double blind placebo controlled, randomized, single cross-over study to evaluate the potential anti-oxidant activity of Niaspan® (EXPLORE)

5.1.1 Introduction

Niacin (nicotinic acid or vitamin B3) is a water soluble vitamin that at physiologic concentrations is a substrate for the synthesis of NAD and NADP, which are important cofactors in intermediary metabolism (the intracellular process in which nutrient molecules are metabolized and converted into cellular components). The pharmacologic effect of niacin requires large doses (1.5-3 g/day) and is independent of the use of nicotinic acid to produce NAD or NADP. Niacin has been used as a pharmacologic agent to regulate abnormalities in plasma lipids and lipoprotein metabolism since 1955.[413]

Niacin is the most effective commercially available agent to increase HDL-C levels.[416] Indeed, a meta-analysis of more than 80 clinical trials revealed that niacin (all formulations) induced elevation in HDL-C levels to a degree that was 1.6-fold higher than that achieved with fibrates.[392] Furthermore, niacin not only reduces elevated TG levels by 15 % to 40 % as a function of baseline levels, but also reduces LDL-C by up to 25 %; the magnitude of the decrement in LDL-C varies with the formulation and dose employed.[653]

Niaspan®, an extended-release formulation of niacin, reduces the incidence of niacin-induced major side effects: flush and hepatotoxicity. Although its mechanisms are not fully understood, the effects of Niaspan® are thought to be due to an increase Apo A-I levels, a reduction in the rate of hepatic uptake of HDL particles, a shift in the distribution of the HDL subfractions, with and increase of HDL2 when compared to HDL3.[422]

Data regarding the effect of extended-release niacin on ApoA-I metabolism are controversial. Niacin has been shown to increase the production rate of ApoA-I both in the liver and in the intestinal cells.[422,424] In fact, niacin activates both MAP kinase and the PPAR transcription factors pathways, which affect ApoA-I production.[112] However, *in vitro* studies using hepatic cells and animal experiments with mice reported no effect of niacin on ApoA-I production rate, but a decreased in ApoA-I hepatic clerarance.[654,655]

Furthermore niacin is the only commercially available agent known to specifically reduce circulating levels of Lp(a); this decrement may reach 30% or more in certain instances.[656] Lp(a) is similar to LDL, but contains apo (a) in addition to ApoB-100 in its protein moiety. It plays an intricate role in the development of atherothrombosis and premature CHD when present at elevated plasma levels (>50 mg/dL), and/or when associated with elevated levels of LDL-C (>130 mg/dL).[657]

Despite being proven that extended-release niacin increases HDL-C levels in plasma, there are no studies regarding its effect on HDL function.

5.1.2 Aim

The aim of this study was to assess qualitative changes in the anti-oxidant activity of HDL, as well as in the titres of IgG aHDL and aApoA-I antibodies independently of the plasma reduction of the lipoprotein levels or HDL-C increase in patients treated with extended-release niacin.

5.1.3 Patients and methods

Study design

This exploratory phase II, randomized double blind placebo controlled, single cross-over study (EMR 64300-609) was investigator initiated and conducted in one center in Portugal: Department of Pharmacology, Faculty of Medical Sciences, Lisbon, Portugal.

The study was divided in two sequential treatment periods that had the same scheduled visits and assessments (Figure 48). The first treatment period started at V1 (week1) and the subjects eligible for enrolment had to meet all the protocol inclusion criteria and none of the exclusion criteria; in Visit 1 patients were randomized to placebo or Niaspan® for a total of twelve weeks. The first 7 weeks of each treatment period corresponded to the titration dose period, which was followed by a period of 5 weeks on maximum dose (1500mg of Niaspan®

or placebo). After 4 weeks of wash-out period and cross-over of treatment arms, Niaspan® or placebo were provided for an equivalent period (second treatment period). After a follow-up period (4 weeks after last visit of the second treatment period) a final assessment of safety and efficacy was performed.

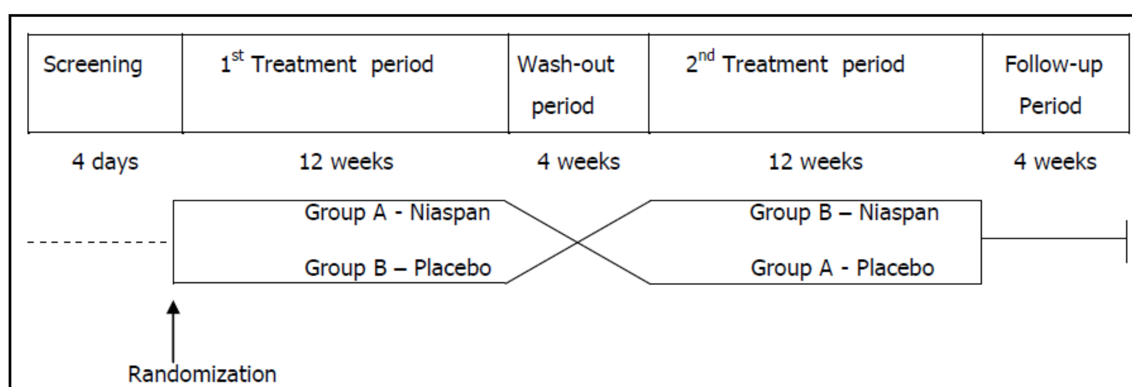


Figure 48. Study design scheme.

The study had a screening visit up to 4 days before randomization (these two visits could be combined in the same evaluation day if all assessments required for the study were available). The first period of treatment included scheduled visits at weeks 1, 4, 8 and 13. Followed by a wash-out period and cross-over of the treatment groups that was scheduled for visit at week 17. The second period of treatment included scheduled visits at weeks 20, 24 and 29. The follow-up period and final visit were scheduled for week 33.

Niaspan® or placebo was administered orally once a day, at night. Subjects had an initial loading dose from weeks 1 till 8 (500 mg and 1000 mg daily), followed by a total of 5 weeks with maximum (1500 mg) dose, corresponding to the first period of treatment. After 4 weeks of wash-out and cross-over of treatment arms, Niaspan® or placebo were provided for an equivalent period (second period of treatment).

Study subjects

Patients were screened in 2009 and men and nonpregnant women who were ≥18 years of age and with HDL-C ≤ 40 mg/dL in men or HDL ≤ 50 mg/dL in women and were included in this trial. No other dyslipidemic medication besides the study medication was used during the study course.

Exclusion criteria included a history of sensitivity to any of the components of the treatment drug; serious or unstable medical or psychological conditions that could compromise the patient's safety or successful trial participation; treatment with statins,

fibrates or anti-oxidant during the 8 weeks prior to enrolment; serum LDL-C > 180 mg/dL and TG > 200 mg/dL; clinically relevant CAD, type 2 diabetes or other relevant CVD; a history of active peptic ulcer; a history of active arterial hemorrhagic; a history of drug abuse (with the exception of alcohol abuse); creatinine clearance > 60 mL/min; total bilirubin ≥ 2 times the upper limit of normal; AST and ALT ≥ 3 times the upper limit of normal; and participation in another investigational drug trial within 30 days of trial enrolment.

All participants gave informed consent before any trial procedure was initiated. The relevant institutional review boards approved the trial protocol and any amendments, and the trial was performed in accordance with the principles of the Declaration of Helsinki and according to Good Clinical Practice guidelines.

Assessments

Blood assessments were carried out in the fasting state at baseline (before randomization to Niaspan® or placebo) and for the safety parameters (hematology and clinical chemistry) at week 17 and 33, and for target parameters at week 8, 12, 17, 24, 29 and 33. The target parameters included PON1 activity, serum nitric oxide metabolites (NO_2^- and NO_3^-), total HDL-C, HDL sub-fractions 2 and 3, ApoA-I levels, IgG aHDL and aApoA-I antibodies

Biochemical parameters

Serum IgG aHDL and aApoA-I antibodies titters were measured by ELISAs and ApoA-I, total HDL-C, HDL2 and HDL3, PON1 activity and nitric oxide metabolites (NO_2^- and NO_3^-) levels were tested as described in methods.

Statistical analysis

The primary efficacy end point was the change of PON1 activity in serum and the analysis was performed based on the intention-to-treat and per protocol populations. Changes of PON1 activity from baseline to the end of the treatment periods were analyzed (within and between treatment groups), using Wilcoxon test. Mean changes and 95% confidence intervals were presented.

Secondary efficacy endpoint included changes in serum nitric oxide metabolites, lipid profile and IgG aHDL and ApoA-I antibodies, and were analyzed within and between treatment groups using Wilcoxon test. The secondary efficacy analysis was performed based on the intention-to-treat population.

Pearson coefficients were used to analyze the correlation between changes in HDL-C and/or ApoA-I levels and the following variables: PON1 activity, serum nitric oxide metabolites levels and IgG aHDL and aApoA-I antibodies.

Sample size was calculated with SD for change in log-transformed PON1 activity values of 0.34 from a previous study. With a power of 80% , it was estimated that a total of 30 patients needed to be randomized.

Thus, assuming a 15% dropout rate, a minimum of 36 subjects were planned to be randomized.

Quantitative variables were described using mean, SD, median, range and 95% confidence interval when applicable. Qualitative variables were summarized by number of observations (n) and percentages (%). All statistical tests were two-tailed with a significance level of 5%.

Statistical analysis was performed using SPSS (Version 21.0) software package.

Safety assessments included recording of treatment-emergent adverse events (adverse events that started or worsened during randomized treatment), hematologic and clinical chemistry measurements (performed in the same central laboratory), and physical examinations. All patients who received any study drug were included in the safety analysis, and safety data were summarized descriptively without statistical analysis.

5.1.4 Results

Study population and baseline characteristics

A total of 21 patients were enrolled as the study was interrupted earlier due to a lack of efficacy. Of the 21 patients enrolled and randomized, 4 patients discontinued treatment due to the occurrence of adverse events (2 from Niaspan® group and 2 from the placebo group). Since these 4 patients left the study before the occurrence of cross-over, only 17 patients completed and were taken into account for the efficacy analysis.

The baseline characteristics are summarized in Table 14 and were similar in both groups. There were 44% and 50% of patients, respectively in group placebo and Niaspan® who had a prior history of hypertension. The baseline lipid profile was comparable amongst the groups.

Table 14. Baseline demographic characteristics and clinical and serological data of two groups

	Placebo (n = 9)	**Niaspan® (n = 8)**	**p**
Age (years)	49 ± 13	47 ± 11	0.57
Sex: female/male (n)	4 / 7	3 / 7	1.00
Ethnic origin: black/caucasian (n)	1 / 8	1 / 7	1.00
Weight (Kg)	78.2 ± 13.8	87.9 ± 17.5	0.22
Height (m)	1.69 ± 0.09	1.77 ± 0.11	0.12
BMI (Kg/m^2)	29.6 ± 4.6	28.6 ± 4.0	0.64
SBP (mmHg)	124.1 ± 17.1	133.5 ± 17.2	0.27
DBP (mmHg)	76.4 ± 12.9	79.8 ± 7.5	0.52
Medical History, N (%)			
Hypertension	4(44)	4 (50)	1.00
Cerebrovascular disease	0(0)	1 (12)	0.47
Others	5(56)	7(87)	0.29
Lipid profile (mg/dL)			
LDL-C	132.5 ± 31.2	141.5 ± 25.4	0.43
Total cholesterol	199.8 ± 27.6	206.2 ± 28.6	0.51
TG	127.5 ± 52.7	151.9 ± 67.9	0.25
VLDL-C	25.5 ± 10.5	30.5 ± 13.6	0.23
HDL-C	35.3 ± 7.7	34.3 ± 4.6	0.63
HDL2	7.4 ± 3.0	6.4 ± 1.7	0.23
HDL3	27.9 ± 5.2	27.9 ± 3.7	0.98
ApoA-I	135.9 ± 18.2	133.7 ± 11.6	0.69
Biological variables			
PON1 activity (U/L)	333.1 ± 101.8	331.3 ± 97.9	0.70
$NO_2^- + NO_3^-$ (μM)	58.9 ± 37.0	55.8 ± 38.8	0.82
IgG aHDL (% p. control)	126.7 ± 67.2	128.3 ± 102.1	0.44
IgG aApoA-I (μg/mL)	0.37 ± 0.14	0.34 ± 0.10	0.55

Abbreviations: Body mass index (BMI); systolic blood pressure (SBP), diastolic blood pressure (DBP), Low density lipoprotein-cholesterol (LDL-C); Triglycerides (TG); Very low density lipoprotein-cholesterol (VLDL-C); High density lipoprotein-cholesterol (HDL-C); Apolipoprotein A-I (ApoA-I); Paraoxonase 1 (PON1); nitric oxide metabolites ($NO_2^- + NO_3^-$); anti-HDL antibodies (aHDL) and anti-ApoA-I antibodies (aApoA-I). Data were presented as mean ± SD. Differences between means were evaluated using Mann-Whitney t-test.

Flushing was reported as moderate/mild by 3 (37.5%) subjects in the extended-release niacin group and 1 (11.1%) in the placebo group, but none stopped the medication. Others adverse events include epigastric pain, dry mouth and headache also occurred at similar rates in both groups.

No significant differences were observed in the safety hematologic and clinical chemistry parameters assessed (transaminase, total bilirubin, creatinine, creatine kinase, electrolytes and uric acid) between the two study groups.

Effect of Niaspan® on lipid profile

Patients treated with extended-release niacin showed a decrease of VLDL-C (p = 0.04), TG (p = 0.04) and increase of HDL2 (p = 0.04) levels when compared to placebo at the end of treatment (Figure 49 A and B).

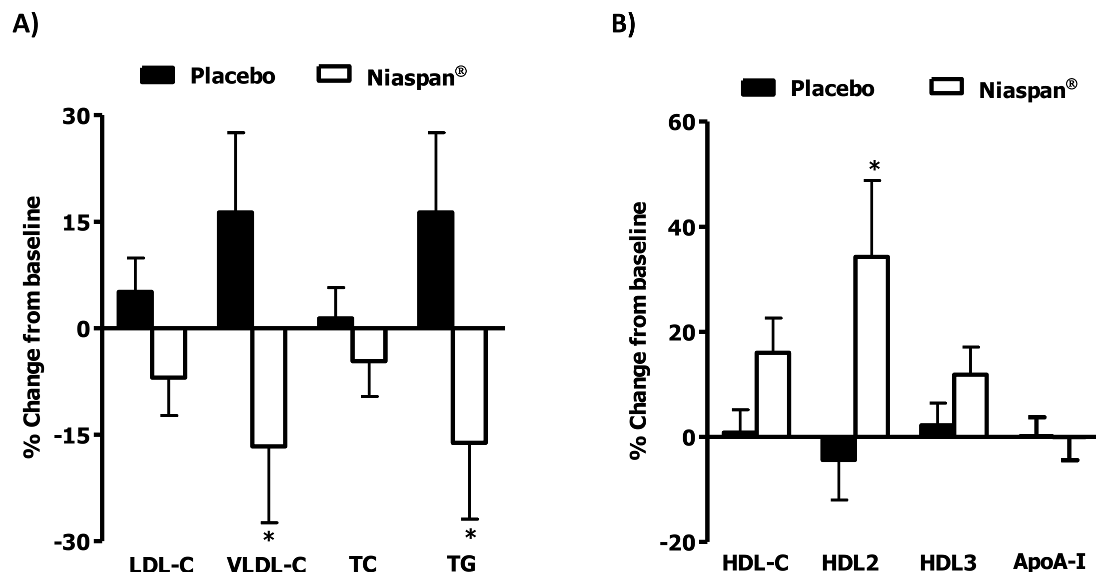


Figure 49. Effect of treatments on lipid profile. Percentage of change from baseline on low density lipoprotein-cholesterol (LDL-C), very low density lipoprotein-cholesterol (VLDL-C), total cholesterol (TC) and triglycerides (TG) levels at the end of 12 weeks of treatment (A). Percentage of change from baseline on total high density lipoprotein-cholesterol (HDL-C), HDL2, HDL3 and apolipoprotein A-I (ApoA-I) levels at the end of treatment (B) Bars show the means. Black bars represents placebo group and white bars the Niaspan® group. Differences between means were evaluated using Mann-Whitney t-test

Although LDL-C, total cholesterol, HDL-C and HDL3 levels did not differ between treatment groups in a statistically significant fashion, the levels of LDL-C, total cholesterol decrease and HDL-C and HDL3 increase when compared to placebo at the end of treatment

Moreover, the levels of total HDL-C and HDL3 subclass were positively associated with ApoA-I levels in both groups of study both at baseline and at the end of treatment (r = 0.73, p = 0.001 and r = 0.74, p = 0.0009, respectively). The HDL2 subclasses also showed a positive association with ApoA-I levels but only in placebo group at baseline and in extended -release niacin group at the end of treatment (r = 0.72, p = 0.001 and r = 0.65, p = 0.005, respectively).

Effect of Niaspan® on PON1 activity

PON1 activity of both groups was comparable at the baseline and no significant effect on PON1 activity was observed after extended-release niacin treatment in comparison to placebo (Figure 50).

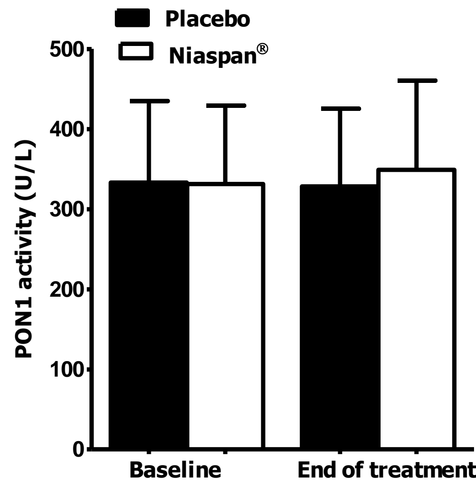


Figure 50. Paraoxonase 1 (PON1) activity acording treatment groups at baseline and at end of 12 weeks of treatment. Bars show the means ± SD. Black bars represents placebo group and white bars the Niaspan® group.

No association was found between PON1 activity and the levels of total HDL-C, HDL2, HDL3 subclasses and ApoA-I in both groups at baseline or at the end of treatment.

Effect of Niaspan® on nitric oxide metabolites

No significant difference were observed on total nitric oxide metabolites ($NO_2^- + NO_3^-$) levels between both treatments at the end of study (Figure 51), nor between mean NO_2^- and NO_3^- levels.

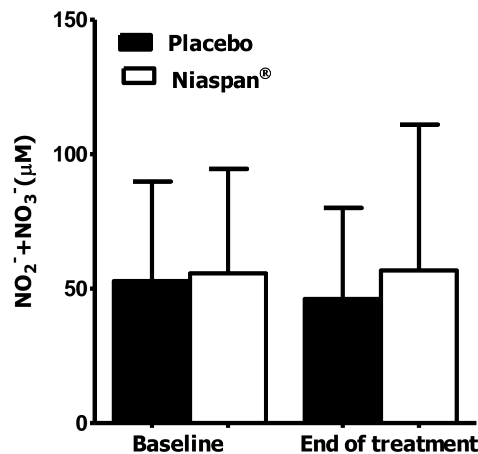


Figure 51. Serum nitric oxide metabolites ($NO_2^- + NO_3^-$) acording treatment groups at baseline and at the end of 12 weeks of treatment. Bars show the means ± SD. Black bars represents placebo group and white bars the Niaspan® group.

Effect of Niaspan® on titres of IgG aHDL and aApoA-I antibodies

Baseline IgG aHDL and aApoA-I antibodies titres were non-significantly different in the two groups of study. The level of IgG aApoA-I antibody increased 73% after 12 weeks of

treatment with extended-release niacin in comparison with placebo (p = 0.001) (Figure 52). There was no significant variation regarding IgG aHDL antibodies in either groups.

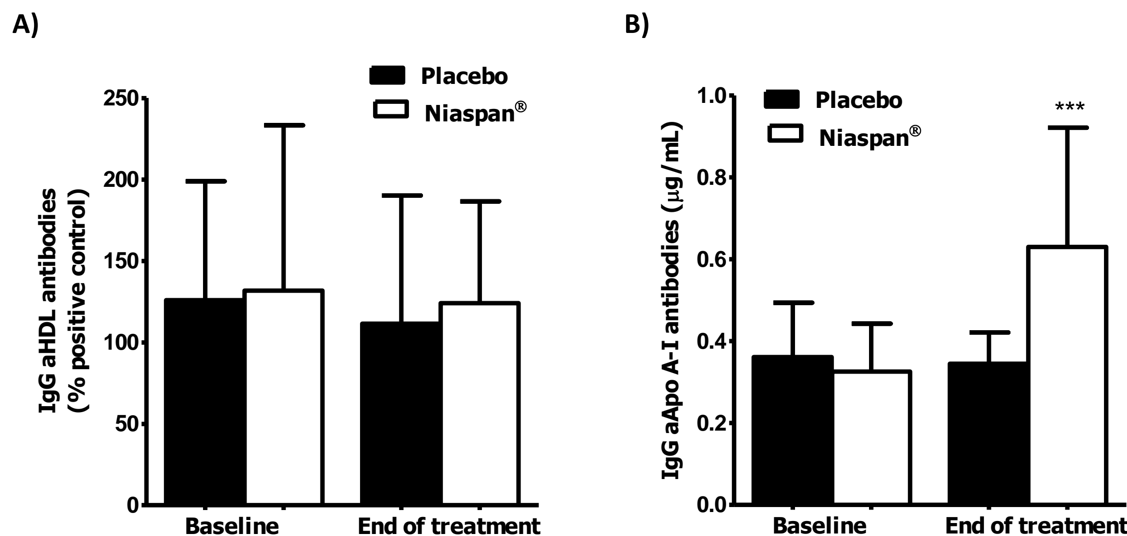


Figure 52. Levels of IgG aHDL (A) and IgG aApoA-I (B) antibodies in both groups of study at baseline and at the end of 12 weeks of treatment. Bars show the means ± SD. Black bars represents placebo group and white bars the Niaspan® group. Differences between means were evaluated using an Mann-Whitney t test. ***p=0.009 represents the statistical comparison between Niaspan® and placebo group at the end of 12 weeks of treatment.

Although titres of IgG aHDL antibodies did not differ between treatment groups, there was a positive correlation between IgG aHDL and aApoA-I antibodies after 12 weeks of treatment with extended-release niacin (p = 0.03) (Figure 53 A). A positive correlation was observed between HDL2 and IgG aApoA-I antibodies (p = 0.03) after the 12 weeks of treatment with extended-release niacin, that was not observed at baseline, neither in the placebo group (Figure 53 B).

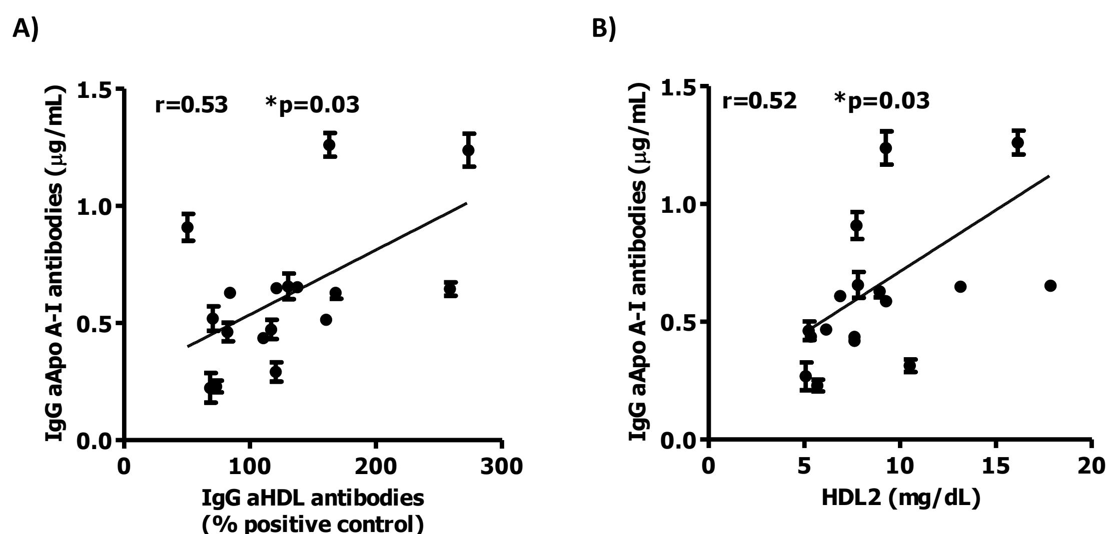


Figure 53. Correlation (Spearman's rank test) between IgG aApoA-I and IgG HDL antibodies (A) and HDL2 levels (B) at the end of treatment in subject taking extended-release niacin.

Despite the fact that treatment did not alter PON1 activity a negative correlation was observed between PON1 activity and IgG aApoA-I antibody titres at baseline and at the end of treatment, in the group of subjects taking extended-release niacin (p = 0.02 and p = 0.01, respectively) (Figure 54).

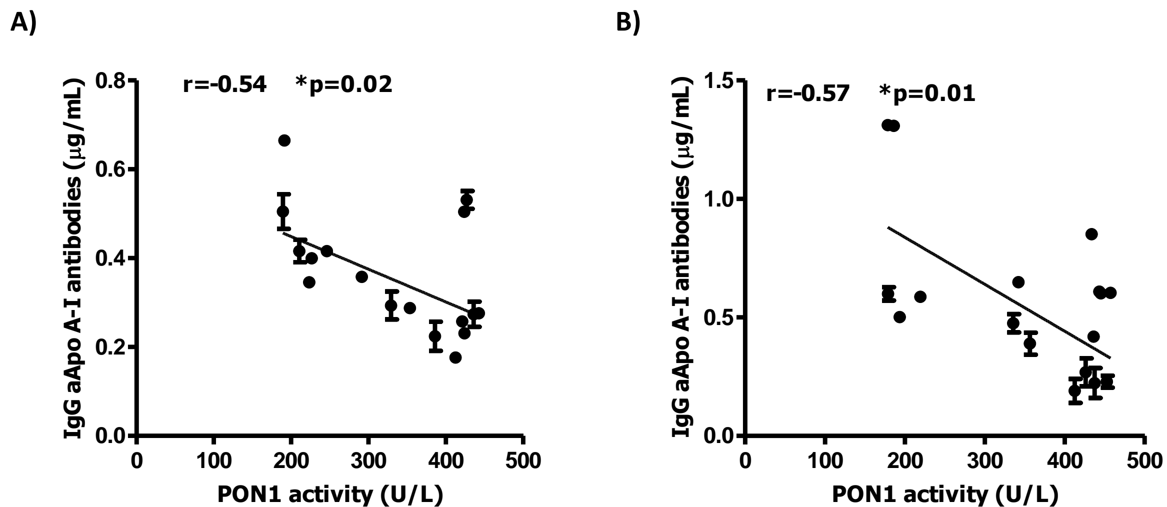


Figure 54. Correlation (Spearman's rank test) between IgGaApoA-I antibodies and PON1 activity at baseline (A) and at the end of 12 weeks of treatment (B) in subject taking extended-release niacin.

5.1.5 Discussion

In this study levels of VLDL-C, TG decreases and HDL2 subclasses increased after treatment with extend release niacin when compared to placebo, however PON1 activity did not significantly increased. The main findings of this study was the enhancement of IgG aApoA-I antibodies at the end of 12 weeks of treatment with extend release niacin when compared with the placebo group. This results suggest that despite the concentration of HDL2 increase its protective capacity or functionality does not increase with extend release niacin, rather these increases resulted in a enhancement of the production of auto-antibodies against HDL components.

Previous studies suggest that bedtime niacin administration diminishes lipolysis and release of free fatty acids to the liver; this, in turn, leads to an abolition of the usual diurnal increase in plasma TG, which may result in diminished formation and secretion of TG in the VLDL fraction.[417,418] It has been proposed that alterations in VLDL-C production by the liver limits plasma CETP activity which exchanges TGs in VLDL and LDL particles for cholesteryl esters in HDL particles, thus a reduction in free fatty acids could explain, at least in some part, the effects of niacin on VLDL-C, HDL-C and LDL-C. This study confirms the effect of extended-release niacin in reducing the TG, VLDL-C levels and increasing HDL2 subclasses.

Although treatment with extended-release niacin did not changed LDL-C, total cholesterol, HDL-C and HDL3 levels in a statistically significant fashion, the levels of LDL-C, total cholesterol decrease and HDL-C and HDL3 increase when compared to placebo. Moreover treatment with extended-release niacin did not change the ApoA-I levels as previously descried.[422,424]

The lipid-modifying efficacy of niacin requires a minimum dose of 1000 mg/day, with the greatest lipid efficacy seen at the 2000 mg/day dose. Progressive increase in the dose of Niaspan® tablets by 500 mg/day at monthly intervals produced incremental reductions in LDL-C and ApoB levels each of approximately 5%, together with incremental increases in HDL-C of approximately 5% over the dose range of 500 mg to 2000 mg/day.[658]

In the present study the extend release niacin dose was escalated to 1500 mg/day in all patients that completed the study. The effect of the doses of 1500 mg, 1000 mg and 500 mg/day of extend release niacin, when analyzed separately showed that total HDL-C, HDL2, HDL3 and ApoA-I elevation were more marked in the lower dose group. Thus, the effect on total HDL-C, HDL2, HDL3 or ApoA-I by extend release niacin was found not to be dose dependent, in this study.

A recent study comparing the effect of niacin and ezetimebe on serum paraoxonase and arylesterase activity showed a significant reduction in both PON activities with ezetimide, but no change was observed in PON or arylesterase activity with niacin.[659] Our study had similar results with no significant differences on PON1 activity after treatment with extended-release niacin or placebo.

Furthermore, niacin also improves endothelial dysfunction, measured by flow-mediated dilation (FMD) of the brachial artery and nitroglycerin-mediated endothelium-independent dilation (NMD), in patients with CAD and low HDL-C, but not with normal HDL-C.[432] In addition, extended-release niacin therapy improved the capacity of HDL to stimulate endothelial NO•, to reduce superoxide production, and to promote endothelial progenitor cell–mediated endothelial repair in patients with type 2 diabetes.[431] The present study did not show an increase in NO• production with niacin-treatment.

The effect of lipid modifying agents on antibodies toward HDL and its constituents had never been study. Interestingly, titres of IgG aApoA-I antibodies were significantly higher after 12 weeks of treatment with extended-release niacin in comparison with placebo. Although titres of IgG aHDL antibodies were not increased, they were positively associated

with IgG aApoA-I antibodies. Furthermore, HDL2 was positively associated with IgG aApoA-I antibodies and PON1 activity was negatively associated on the group treated with extended-release niacin.

All together this data suggest that extended-release niacin may increase the levels of HDL2 subclasses but it did not improve the functionality of HDL. In fact, by increasing titers of IgG aApoA-I antibodies niacin may be is to increase the dysfunctionality HDL.

Nonetheless, there are several limitations to this study. First of all, this study had a small number of patients, the wash-out period of 4 weeks may not have been sufficient for the crossover of the treatments and the duration of treatment of 12 weeks may not have been sufficient. Finally, patients included in this study did not have an elevated cardiovascular risk, with exception of low HDL-C levels, but normal ApoA-I levels.

Niacin is the most effective medication in current clinical use for increasing HDL-C, however if this increase is translated in a increase of HDL function is not clear. This study suggests that the anti-oxidant properties of HDL are not induced by extend release niacin. Due to the already referred study limitations, these results should be viewed as hypothesis-generating and will require replication in other study.

5.2 Prevention of atherosclerosis with atorvastatin in patients with systemic lupus erythematosus (SLE): a pilot study

5.2.1 Introduction

CVD accounts for a significant morbidity and mortality in patients with SLE[594], with a frequency of 6.1 to 8.9 (varies with age) fold higher than in the normal population, even after correction for the traditional Framingham risk factors.[660] Age at first MI occurs almost 20 years earlier than in the general population.[661]

An association of atherosclerosis with disease-specific factors, such as prolonged steroid treatment, chronic inflammation and renal disease has been demonstrated in this population,[595-597] together with an atherogenic lipid profile, characterized by elevated levels of VLDL, TG and lower levels of HDL.[598]

Statins have been used extensively in clinical practice to reduce cardiovascular-related morbidity and mortality. This effect is partly mediated through beneficial effects on the lipid profile, in particularly lowering LDL-C, but accumulated evidences suggested that statins

exert immunomodulatory and anti-inflammatory actions independently of their lipid-lowering effects.[662,663] These pleiotropic immunomodulatory properties of statins indicate that theses might have a therapeutic effect on autoimmunity and atherosclerosis, acting in these was on both factors for CVD in patients with SLE. Unfortunately, very few observational and interventional trials have evaluated the effect of statins in SLE, which makes routine statin therapy inappropriate in these patients

5.2.2 Aim

This study aimed at determining the effect of one year placebo controlled pilot trial with atorvastatin in some pro-atherogenic oxidant and inflammatory markers, as well as in the titres of antibodies towards HDL complex on SLE patients.

5.2.3 Patients and methods

Patients and study design

The study was designed as a pilot, randomised, double blind and placebo controlled. SLE patients were stratified by age (age <40 or >40 years) and randomised by computer generated numbers in a 1:1 ratio to receive 40 mg of oral atorvastatin daily or placebo. Between September 2006 and November 2008, consecutive SLE patients from the Leeds Connective Tissue Disease Clinic, who fulfilled the inclusion and exclusion criteria, were invited to participate in the study.

Inclusion criteria were 4 or more the ACR revised criteria[587,588] for the classification of SLE, being 18 years of age, able to understand and provide informed consent and willingness to switch to atorvastatin if already on a different statin, unless contraindicated.

Exclusion criteria were inability or unwillingness to give informed consent, acute or chronic infections, uncontrolled heart failure, uncontrolled endocrine disease, uncontrolled hypertension, mal-absorption, acute liver disease, heavy alcohol intake, pregnancy or risk of pregnancy (not using appropriate contraception or planning pregnancy over the study period), breast feeding and previous statin intolerance.

The relevant hospital committee granted ethical approval (reference MREC 04/012). Patients were seen at baseline and then every 3 months for one year. At baseline case notes were reviewed with regards to traditional CVD risk factors, past and current medication, history of vascular occlusions (ischemic heart disease with or without MI, IS, peripheral vascular disease). At each visit, including baseline, patients underwent a physical

examination and had their weight and blood pressure checked and women had a pregnancy test. At each visit routine blood samples were taken for full blood count, clotting screen including lupus anticoagulant, renal and liver function tests, lipid profile (TG, total cholesterol, LDL-C and HDL-C), IgG aCL. In addition research blood samples were taken in baseline and after one year of treatment for, -hsCRP, SAA, oxLDL β2GP1, nitric oxide metabolites ($NO_2^- + NO_3^-$), plasma 3-NT, TAC, PON1 activity and IgG aHDL and aApoAI antibodies.

Forty four patients were enrolled in the study. Of these 7 were already on a different statin and were switched to atorvastatin; therefore 37 SLE patients were randomized, 20 in the placebo arm and 17 in the atorvastatin arm. After randomisation 7 patients from the placebo arm and 7 from the atorvastatin arm dropped out or simply ceased attending. Final pre and post data were available for 17 patients in the atorvastatin arm, of whom 12 were statin naïve and 5 were statin pre-treated (simvastatin n=4, pravastatin n=1; switched to atorvastatin at study entry) and for 13 patients in the placebo arm.

Biochemical parameters

Serum IgG aHDL and aApoA-I antibodies titers were measured by ELISAs and plasma levels of 3-NT, hs-CRP and SAA, oxLDLβ2GPI complex and serum PON1 activity, TAC and nitric oxide metabolites ($NO_2^- + NO_3^-$) levels were quantified as described in methods.

Statistical analysis

For categorical variables, Chi-squared test or Fisher's exact test were used. Univariate comparisons between baseline and one year of atorvastatin treatment for continuous variables was carried out by Mann-Whitney t-test paired. Statistical analysis was performed using the GraphPad Prism software, version 5 (GraphPad Software Inc., San Diego, USA).

5.2.4 Results

Characteristics of the study subjects

Baseline demographic and clinical characteristics of the placebo and atorvastatin groups were matched (Table 15).

Regarding the biological variables measured all of them were comparable among the groups at baseline.

Table 15. Demographic characteristics, clinical and laboratory data of patients with systemic lupus erythematosus (SLE) randomized to placebo and atorvastatin group.

	Placebo (n = 13)	**Atorvastatin (n = 17)**	**p**
Age (years)	48 ± 10	55 ± 10	0.08
Sex: female/male (n)	13/0	17/0	1.00
Ethnic origin: black/caucasian (n)	3 / 11	0 / 17	0.07
Disease duration (years)	15 ± 11	9 ± 8	0.10
BMI (Kg/m^2)	26.4 ± 4.0	26.5 ± 5.4	0.93
SBP (mmHg)	128.0 ± 21.2	125.0 ± 14.4	0.65
DBP mmHg)	79.0 ± 3.5	82.5 ± 24.8	0.62
Medical History N (%)			
Hypertension	8 (61.5)	11 (64.7)	1.00
Type 2 diabetes	0	1 (5.9)	1.00
Dyslipidemia	3 (23.1)	6 (35.2)	0.69
Medication N (%)			
Prednisolone	7 (53.8)	11 (64.7)	0.71
Hydroxycloroquine	7 (53.8)	8 (47.0)	1.00
Methotrexate	2 (15.4)	3 (17.6)	1.00
Anti-hypertensive agents	7 (53.8)	9 (52.9)	1.00
Warfarin	1 (7.7)	4 (23.5)	0.35
Aspirin	8 (61.5)	4 (23.5)	0.06
Laboratory features N (%)			
Ig aCL > 20GPL	3 (23.1)	1 (5.9)	0.29
Lupus anticoagulant	2 (15.4)	1 (5.9)	0.56

Abbreviations: Body mass index (BMI), systolic blood pressure (SBP); diastolic blood pressure (DBP); anticardiolipin (aCL); Data were presented as mean ± SD.

Changes in measured biological variables over 12 months

After one year of therapy with atorvastatin patients with SLE showed a significant decreases in total cholesterol (-22%), LDL-C (-37%), hs-CRP (-75 %) and oxLDL β2GP1 (-18 %) levels, in comparison with the placebo group (Table 16).

No differenced were observed in SAA levels between atorvastatin and placebo group. 3-NT levels significantly increased in placebo group (+82%) in comparison with atorvastatin group.

Non-significant increases were observed in HDL-C (+0.3%), TAC (+6%) and PON1 activity (-6%) even decreased after on year of treatment with atorvastatin.

No statistical significant differences in the IgG aHDL and aApoA-I antibodies titres were observed after on year treatment in comparison with placebo, nevertheless the increase of the IgG aApoA-I antibodies in placebo group when compared with baseline was higher than in atorvastatin group.

Table 16. Biological variables mesuared of patients with systemic lupus erythematosus (SLE) randomized to placebo and atorvastatin group.

	Placebo (n=14)			Atorvastatin (n=17)			p
	Baseline	After 1 year	Δ%	Baseline	After 1 year	Δ%	
Total cholesterol (mg/dL)	186.9±66.5	212.7±39.4	+14	174.5±	3.2±0.6	-22	0.0008
LDL-C (mg/dL))	96.4±48.9	122.3±20.5	+27	96.4±37.7	60.7±23.12	-37	<0.0001
HDL-C (mg/dL)	67.5±17.5	69.8±17.7	+3	56.9±26.5	57.1±19.9	+0.3	0.412
TG (mg/dL)	100.8±59.9	103.3±42.0	-3	103.3±42.0	93.2±47.4	-10	0.419
hs-CRP (μg/mL)	3.1±2.8	2.8±1.9	-9.	5.1±7.4	1.4±1.0	-75	0.001
SAA (μg/mL)	19.7±6.7	3.5±2.4	-82	19.5±10.0	4.7±4.3	-76	0.417
oxLDLβ$_2$GPI (units/mL)	2.7±0.6	3.1±0.6	+14	2.6±0.6	2.1±0.7	-18	0.003
NO_3^- (μM)	32.5±24.5	28.0±18.1	-14	23.9±14.8	21.3±13.9	-16	0.778
NO_2^- (μM)	8.9±4.6	9.3±4.8	+5	11.7±5.9	10.9±4.1	-6	0.675
3-NT (nM)	3.5±8.0	6.6±2.0	+82	4.1±1.9	6.5±4.3	+15	0.005
TAC – Vit E analogue equivalent units (mM)	9.0±2.0	9.5±1.9	+6	9.1±2.1	9.7±1.7	+6	0.769
PON1 activity (U/L)	194.2±57.1	186.9±51.8	-4	203.9±45.4	192.8±45.5	-6	0.900
IgG aHDL antibodies (%p. control)	154.2±80.3	141.0±72.8	-8	110.4±35.8	100.9±51.2	-8	0.933
IgG aApoA-I antibodies (μg/mL)	1.0±0.3	2.1±1.6	+102	1.6±1.3	2.0±1.6	+23	0.072

Abbreviations: Δ%: percentage difference; TG: triglycerides; Low density lipoprotein-cholesterol (LDL-C); High density lipoprotein-cholesterol (HDL-C); hs-CRP: high sensitive C-reactive protein; SAA: serum amyloid A; oxLDLβ2GPI: oxidised low density lipoprotein-beta 2 glycoprotein-I complex; NO_3^-: nitrate; NO_2^-: nitrite; 3-NT: 3-nitrotyrosine; TAC: total antioxidant capacity; PON1: paraoxonase 1; anti-HDL antibodies (aHDL) and anti-ApoA-I (aApoA-I) antibodies. Data are presented as mean ± SD. Differences between Δ% were evaluated using an Mann-Whitney t-test.

5.2.5 Discussion

In this study, levels of total cholesterol, LDL-C, hs-CRP and oxLDL β2GP1 in SLE patients were reduced after one year of atorvastatin treatment. However, no changes were observed in HDL-C, PON1 activity and TAC after on year of treatment with atorvastatin.

The major effect of statins is the reduction of plasma levels of ApoB-containing lipoproteins, principally LDL (20-60%), with a modest raise of HDL-C levels (by 3% to 15%) [199, 371-373].

Atorvastatin, reduces LDL-C from 35 to 61% over the dose range of 10 to 80 mg, as well as total cholesterol (19 to 45%) and TG (12 to 53%).[664] The effect of atorvastatin on HDL-C is varies between unchanged or increases of approximately 2 to 7%.[665] Some studies show that the increase of HDL-C is inversely related to the dose of atorvastatin (in the range of 20 to 80 mg/day), unlike any other statins.[374,375]

Similarly to previous studies, our results show that atorvastatin significantly reduces total cholesterol (22%), LDL-C (37%) and triglyceride (10%) levels, however there were no changes in HDL-C levels.

With regards to the two inflammatory markers evaluated in this study, SAA and CRP, only the latter showed a significant post treatment changes. Even though the levels of SAA have also decreased significantly in relation to the baseline this effect cannot be attributed to treatment with statin because the same percentage decrease was observed in the placebo group.

Patients treated with atorvastatin seems to have lesser increase of 3-NT when compared to the placebo group (14% vs. 82%).

Statins seems to enhance the activity of HDL-associated enzymes as demonstrated by the increase in PON1 activity (5 to 14%).[667-669] In the present study the PON1 activity decreased in both groups, probably due to the lack of change of HDL-C.

PON1 accounts for most of the HDL protective effect against LDL oxidation hence against atherosclerosis itself and its activity can be reduced in SLE as showed in previously chapters. To counteract this mechanism, β2GPI, binds to oxLDL to form the covalent complex oxLDL β2GPI. This complex is elevated in patients with APS, SLE[136] and chronic nephritis.[542] Here we show that atorvastatin treatment induces a reduction of serum concentration of the oxLDLβ2GPI complex in keeping with its anti-atherogenic effect.

Regarding the antibody titres atorvastatin did not have any significant effect, however IgG aApoA-I antibodies increased in both groups though this was more pronounced in the placebo group that also had a lower baseline titre.

5.3 Influence of Rosuvastatin on the oxidative modification of LDL in type 2 diabetes

5.3.1 Introduction

Premature vascular disease is a well-established complication of type 2 diabetes largely promoted by oxidative stress.[27] There is considerable evidence that hyperglycaemia might result directly, or indirectly in the generation of AGEs and/or ROS via the activation of diacylglycerol-PKC pathway in vascular cells, and to increased glucose flux through the aldose reductase pathway.[670] These pathways have been associated with activation of NF-κB

and increased release of $O_2^{\bullet-}$ which play a role in glucose-mediated inflammation and oxidative stress.[671]

Several studies have indicated that patients with type 2 diabetes tend to have more oxidative internal environments than those of healthy normal subjects. Therefore, these patients show an increased ROS generation and oxidative stress markers, such as elevated lipid peroxidation levels with an accompanying decrease in anti-oxidant levels.[672-674]

As in other diseases associated with atherosclerotic cardiovascular complications, enhanced serum levels of oxLDL and oxLDL β2GP1 complexes have been reported.[675,676] In addition, the redox imbalance becomes overwhelmed due to an impaired PON1-mediated HDL anti-oxidant function also observed in these patients.[646,677]

Observations that statins may lower oxLDL β2GP1 complexes[675] and increased PON1 activity[678] may suggest that the pleiotropic effects of this class of drugs may prevent or decrease the oxidative modification of LDL possibly by an antioxidant mechanism. In this study we intended to test this hypothesis; rosuvastatin is a hydrophilic statin with multiple sites that form a strong interaction with the enzyme HMG-CoA reductase and therefore provide more potent enzyme inhibition than other statins.[679] In fact, rosuvastatin has an affinity for the HMG-CoA reductase active site that is 104-fold higher than that of HMG-CoA. In addition, rosuvastatin has an advantage compared to some other statins (like atorvastatin), since it is not metabolized predominantly through CYP3A4, eliminating many potential drug-drug interactions. Furthermore, rosuvastatin together with pitavastatin are the most potent compounds within this class to increase HDL-C (6-12%).[199]

5.3.2 Aim

This study aimed at determining the effect of rosuvastatin in prevent or decrease the oxidative modification of LDL possibly by an antioxidant mechanism mediated by PON1-HDL, as well as in the titres of antibodies towards HDL complex in patients with type 2 diabetes.

5.3.3 Patients and methods

Patients and study design

The study was an open label 2:1 assignment of consecutive diabetes patients into a oral rosuvastatin (10 mg/day for six weeks) arm or an observational arm respectively. Consecutive type 2 diabetes patients attending the endocrinology and diabetes outpatient

clinic at the Hospital General de Occidente, Zapopan, Mexico from August 2007 to October 2008 were invited take part in the study.

The diagnosis of type 2 diabetes mellitus relied on the presence of abnormal FGP (normal range 70–110 mg/dL), abnormal glucose tolerance test, chronic hyperglycaemia and metabolic disturbances of lipid, carbohydrate and protein metabolism due to defects in insulin production or activity.[680]

Exclusion criteria were macroalbuminuria or kidney disease, CVD (MI, angina, peripheral arterial disease and stroke), concomitant systemic acute or chronic inflammatory diseases (bacterial or viral infections), concomitant acute or chronic autoimmune disorders, pregnancy, statin use within 6 months of the study and intolerance to statins.

Diabetes patients with hypertension, obesity, dyslipidemia and microalbuminuria were accepted within the study: hypertension was defined as a systolic blood pressure > 130 mmHg or diastolic blood pressure > 85 mmHg and obesity as a BMI > 27 for the Mexican population.[681] One hundred and twenty five patients were eligible for the study. After the 2:1 assignment, 86 patients entered the rosuvastatin arm and 39 the observational arm. In the rosuvastatin group 10 patients did not complete the study (4 discontinued intervention for minor side effects and 6 did not attend follow-up) and in the observational group 4 did not attend follow-up. The final study included 111 patients of whom 76 completed 6 weeks' treatment with 10 mg daily of oral rosuvastatin and 35 did not receive rosuvastatin and were reviewed at the end of the same time span.

Patients on rosuvastatin were instructed not to modify other regular medications throughout the study. Counselling with a dietician was available to all patients throughout the study.

Physicians involved in the care of the patients were not involved in data analysis and interpretation. The study (#141/07) was approved by the ethics committee of the Hospital General de Occidente, Zapopan (Mexico), performed in accordance with the Declaration of Helsinki and all patients signed informed consent before entering the study.

Fasting blood samples were obtained from all participants at study entry (baseline sample) and from all patients who completed the 6 weeks interventional and observational arms. Sera for routine chemistry, lipid profile, oxLDL β2GPI complexes, nitric oxide metabolites ($NO_2^- + NO_3^-$), 3-NT, ADMA levels, PON1 activity, IgG aHDL and aApoA-I antibodies were stored frozen at -70ºC until tested at the end of the study. Routine

chemistry and lipid profile investigation were performed at the Hospital General de Occidente clinical laboratory in Mexico with standard laboratory methods (Wiener Laboratories, Rosario, Argentina). The measurement of HbA1C was determined by the NycoCard HbA1C (Axis-Shield PoC AS, Oslo, Norway) (reference range 4.5–6.3%). All other parameters were quantified at the Pharmacology Laboratory of Faculty of Medical Sciences of Lisbon.

Biochemical parameters

Serum IgG aHDL, aApoA-I antibodies titers, 3-NT, oxLDL β2GPI complexes and ADMA levels were measured by ELISAs, and serum PON1 activity and nitric oxide metabolites (NO_2^- and NO_3^-) levels were tested as described in methods.

Statistical analysis

For categorical variables, Chi-squared test or Fisher's exact test were used; for continuous variables and to analyse the effect of rosuvastatin treatment on study variables, paired t-test and Wilcoxon Signed Rank Sum test (when applicable) were used. Statistical analysis was performed using the GraphPad Prism software, version 5 (GraphPad Software Inc., San Diego, USA).

5.3.4 Results

Characteristics of the study subjects

Demographics, baseline clinical characteristics and serological data of rosuvastatin and observational arms were shown in Table 17. Both groups were matched on demographic and baseline clinical characteristics. Regarding the serological data all of them were comparable, with exception of total cholesterol and LDL-C levels that were higher on the observational arm.

Table 17. Demographic characteristics, clinical and serological data of patients with type 2 diabetes randomized to receive or not rosuvastatin.

	Type 2 diabetes patients		
	Without Rosuvastatin (n = 35)	**With rosuvastatin (n = 76)**	**p**
Age (years)	56 ± 7	54 ± 12	0.620
Sex: female/male (n)	27/8	53/23	0.499
Disease duration (years)	7.6 ± 6.1	7.9 ± 7.0	0.972
Medical History N (%)			
Hypertension (%)	5 (14.2)	18 (23.7)	0.319
Obesity (%)	22 (62.8)	33 (43.4)	0.237
Medication N (%)			
Oral glucose lowering drugs	30 (85.7)	61 (80.2)	0.600
Insulin	5 (14.3)	13 (17.1)	0.788
Anti-hypertensive agents	6 (17.1)	7 (9.2))	0.339
Serological data			
FGP (mg/dL)	137.1 ± 67.9	160.2 ± 82.8	0.253
HbA1c (%)	7.5 ± 1.9	7.6 ± 2.8	0.927
CRP (mg/L)	2.8 ± 0.3	2.5 ± 0.3	0.191
Total cholesterol (mg/dL)	224.5 ± 22.6	199.5 ± 36.1	0.0002
TG (mg/dL)	227.7 ± 120.3	222.2 ± 125.5	0.529
LDL-C (mg/dL)	141.4 ± 26.7	120.1 ± 31.9	0.001
HDL-C (mg/dL)	45.5 ± 9.3	46.1 ± 9.7	0.717

Abbreviations: Fasting plasma glucose (FPG); Glycosylated hemoglobin (HbA1c); C-reactive proten (CRP) Triglycerides (TG). Data are presented as mean ± SD. Differences between means were evaluated using an Mann-Whitney t-test paired.

Effect of six weeks treatment with rosuvastatin

After six weeks of therapy with rosuvastatin, patients with type 2 diabetes showed a significant decreases in total cholesterol (-25%), TG (-27%) and LDL-C (-37%) in comparison with the observational group (Table 18).

Table 18. Effect of six weeks treatment with rosuvastatin in serological data of patients with type 2 diabetes

	Without rosuvastatin (n = 35)		**With rosuvastatin (n = 76)**		
	After 6 weeks	**Δ%**	**After 6 weeks**	**Δ%**	**P**
FGP (mg/dL)	147.5 ± 71.4	+5	154.6 ± 76.0	-3	0.415
HbA1c (%)	7.3 ± 2.0	-3	7.6 ± 2.1	+0.6	0.336
CRP (mg/L)	2.9 ± 0.3	+5	2.4 ± 0.3	-4	0.502
Total cholesterol (mg/dL)	212.2 ± 20.4	-6	150.2 ± 34.6	-25	<0.0001
TG (mg/dL)	210.0 ± 100.2	-8	161.6 ± 78.1	-27	0.021
LDL-C (mg/dL)	126.1. ± 25.3	-12	76.2 ± 34.7	-37	<0.0001
HDL-C (mg/dL)	45.2 ± 9.7	+0.5	45.3 ± 8.7	-1.6	0.726

Abbreviations: Δ%: percentage difference; Fasting plasma glucose (FPG); Glycosylated hemoglobin (HbA1c); C-reactive proten (CRP) Triglycerides (TG). Data are presented as mean ± SD. Differences between Δ% were evaluated using an Mann-Whitney t-test paired.

The rosuvastatin arm showed significant decrements in IgG aApoA-I antibodies (-5.%), NO_3^- (-33.5%) and NO_2^- (-25.5%) levels in comparison with the observational group.

Nevertheless the decrease of oxLDL β2GPI complexes and 3-NT levels were more marked on patients treated with rosuvastatin it was not statistical significant in comparison with observation arm. No changes were noted in the others biological variables analyzed (Table 19).

Table 19. Biological variables mesuared after treatment with or without rosuvastatin on patients with type 2 diabetes.

	Without rosuvastatin (n = 35)			With rosuvastatin (n = 76)			
	Baseline	**After 6 weeks**	**Δ%**	**Baseline**	**After 6 weeks**	**Δ%**	**P**
oxLDLβ$_2$GPI (units/mL)	0.83±0.6	0.76±0.8	-8.3	0.79±0.5	0.53±0.3	-32.6	0.248
PON1 activity (U/L)	205.1±70.6	206.5±78.7	+0.5.	176.4±71.1	176.8±76.9	+0.2	0.880
IgG aHDL antibodies (%p. control)	130.2±62.3	125.6±47.6	-3.5	132.6±51.1	124.3±43.8	-6.3	0.953
IgG aApoA-I antibodies (µg/mL)	0.91±0.4	1.3±0.8	+40.5	1.1±0.7	1.0±0.6	-5.0	0.03
NO_3^- (µM)	33.8±28.2	32.8±22.7	-3.1	53.7±32.2	35.7±13.9	-33.5	0.006
NO_2^- (µM)	20.2±12.2	20.8±14.2	+3.2	23.5±13.8	17.5±10.5	-25.5	0.043
3-NT (nM)	8.3±10.4	8.5±9.7	+1.8	9.9±10.8	8.1±9.2	-17.9	0.142
ADMA (µM)	0.56±0.1	0.53±0.1	-4.7	0.59±0.1	0.56±0.1	-4.6	0.422

Abbreviations: Δ%: percentage difference; oxLDLβ2GPI: oxidised low density lipoprotein-beta 2 glycoprotein-I complex; PON1: paraoxonase 1; aHDL: anti-HDL antibodies; aApoA-I: anti-ApoA-I antibodies; NO_3^-: nitrate; NO_2^-: nitrite; 3-NT: 3-nitrotyrosine; ADMA: Asymmetric dimethylarginine. Data are presented as mean ± SD. Differences between Δ% were evaluated using an Mann-Whitney t-test.

5.3.5 Discussion

The interventional arm of this study showed that 10 mg of oral rosuvastatin daily for 6 weeks was associated with a significant reduction of the IgG aApoA-I antibodies, nitric oxide metabolites levels(NO_2^- and NO_3^-) as well as with the expected decrease in total cholesterol, LDL-C and TG.

Contrary to what has been shown in previous clinical trials we did not observe an increase in HDL-C levels, neither PON1 activity was enhanced by rosuvastatin treatment and therefore could not have contributed its specific antioxidant capacity towards the decrease of oxLDLβ2GPI; likewise CRP did not show a significant decrease after treatment and therefore an anti-inflammatory effect of rosuvastatin seemed less likely.

On the other hand a significant decrease of nitric oxide metabolites (NO_2^- and NO_3^-) may represent a beneficial effect of rosuvastatin as it indirectly indicates a reduction in reactive nitrogen species contributing to LDL oxidation.

In the course of inflammation endothelial and mononuclear cells generate more $O_2^{\bullet -}$ as well as more NO• that behaves as a pathogenic mediator or as a cytotoxic molecule.[625] Most of NO• mediated pathogenicity depends on the formation of secondary intermediates such as $ONOO^-$ and $\bullet NO_2$ that are more reactive and toxic than NO• per se. In fact $ONOO^-$ interacts with CO_2 to give $ONOOCO_2^-$ that will induce nitration of tyrosine residues in proteins.[623] Measurement of nitrated proteins therefore represents a fingerprint of the interaction of $O_2^{\bullet -}$ with NO•.[626] Although rosuvastatin appears to reduce the levels of 3-NT, this reduction was not statistically significant in comparison with the observational arm possibly due to the fact that treatment with rosuvastatin for six weeks was not enough.

Protein and lipid modifications may increase the immunogenicity of intracellular antigens by generations of neo-epitopes, leading to a break in immune tolerance[626] This may be the cause for the production of antibodies toward HDL complex. Another reason may simply be due to an increase in antigen level. Although HDL-C levels did not increased with rosuvastatin treatment, it improved the redox imbalance characteristic of these patients, which may have contributed the decrease in antibody titres towards ApoA-I, in contrast to raise of 40.5% in the observational arm.

An apparent limitation of this study may have been the exclusion of type 2 diabetes patients with cardiovascular, cerebrovascular and peripheral vascular disease and patients with abnormal kidney function and macroalbuminuria, thus our population may have a low degree of *vascular inflammatio*n and therefore marginal or no changes in the markers of inflammation (CRP, ADMA and NT). Another limitation of this study could have been the short period of treatment and the dosage of rosuvastatin that may have limited the effects on HDL.

Our study could not pointedly show that rosuvastatin prevent the oxidative modification of LDL, notably by reducing the oxLDL β2GPI complex concentration and possibly by an antioxidant mechanism mediated by PON1-HDL. Nevertheless, the reduction of the oxidative internal environments by rosuvastatin may have limited the increase of antibodies toward HDL complex demonstrating the benefit of treatment with rosuvastatin.

6. OVERALL DISCUSSION AND PROPOSALS FOR FUTURE DIRECTIONS

A possible humoral response towards HDL has been put forward with this thesis. We first tested this hypothesis in patients with autoimmune disease such SLE, using this disease as a clinical model, due to its enhanced atherogenesis and increased immune (including humoral) activity. We were able to show the presence of both IgG aHDL and aApoA-I antibodies in these patients and showed an association with decreased PON1 activity and TAC, increased biomarkers of endothelial dysfunction (NO• metabolites, adhesion molecules: VCAM-1 and ICAM-1) and an increase in disease-related damage and activity.

Another group has recently confirmed this work and its importance has now been recognised, with the recent suggestion by the American College of Rheumatology that aHDL antibodies could be a new biomarker for vascular disease in the context of systemic autoimmune conditions.[682]

The recognition that the overall background mechanism could be the same outside the spectrum of autoimmune diseases, led us to test whether patients with atherosclerosis-related clinic events such as CAD, IS and type 2 diabetes, would also have these antibodies. Indeed, we found increased titres of IgG aHDL, aApoA-I and aPON1 antibodies in these patients. Moreover, the presence of these antibodies was again associated with a pro-inflammatory and pro-oxidant profile: higher NO•metabolites, VCAM-1, ICAM-1, 3-NT and lower PON1 activity.

Our results suggest that aHDL antibodies were associated with modifications in some known anti-oxidant and anti-inflammatory HDL-related functions. However, these findings were cross-sectional clinical studies, which cannot explain whether the antibodies were causal to the clinical events or whether they were merely an epiphenomenon.

With the overall goal to demonstrate the biologic activity and consequent pathogenic capacity of the antibodies towards the HDL complex we set up a protocol for the antibody isolation from serum of different patients.

These antibodies inhibited PON1 activity in a dose-dependent fashion. Moreover the anti-inflammatory effect of HDL on cytokine-induced production of VCAM-1 was abrogated in more than 80% by aHDL antibodies isolated from patients in an *in vitro* experiment. The aHDL antibodies also abolished the HDL-induced angiogenesis by reducing VEGF levels in 65%. These set of experiments provide evidence for a biologic activity and consequent possible pathogenic potential of these antibodies.

To our knowledge, outside the autoimmune context, only one group has studied aApoA-I antibodies but in a different clinical context. They showed a higher prevalence of aApoA-I antibodies in patients with acute coronary syndrome, which were associated with a decrease of ApoA-I plasmatic levels, an increase of oxLDL levels.[615] They suggested that these antibodies increase the atherosclerotic plaque vulnerability[617] and could be used as a prognostic marker in MI.[616] Their work complemented ours but they have only addressed aApoA-I antibodies in a specific group of acute ischemic heart disease patients.

Finally, we investigated the effect of current available pharmacologic agents for increasing HDL-C concentrations on the antibody titres in comparison with placebo in double blind randomized trials with extended release niacin and atorvastatin, and an open label study with a rosuvastatin arm and an observational arm. It should be noted that none of the three trials was designed for the study of a direct effect on the antibodies towards HDL complex titres as the primary endpoint (except for the trial with extended release niacin).

Niacin is the most effective medication in current clinical use for increasing HDL-C, however it is not clear that this increase is translated into a raise of HDL functions. Our results suggest that the extend release niacin may increase levels of total HDL-C or HDL subclasses but it did not induce an enhancement of HDL anti-oxidant properties and improvement of endothelial dysfunction mediated by HDL. In fact, by increasing the titres of aApoA-I antibodies, niacin may hamper the protective effect of HDL.

Statins only modestly raise HDL-C levels and even then there are differences between their potency to increase HDL-C.

Our results do not show an increase of HDL-C levels by atorvastatin neither rosuvastatin. However both statins were associated with a reduction of serum oxidative modification of LDL notably by reducing the oxLDL β2GPI complex concentration. This antioxidant effect was not through a PON1-mediated mechanism. Nevertheless, the reduction of the oxidative internal environment by atorvastatin and rosuvastatin may have limited the increase of aApoA-I antibodies. Even so, our data seems to suggest that rosuvastatin may potential be more beneficial that atorvastatin in preventing the increase in antibody production.

Due to the previously mentioned limitations of our trials some of these results might be viewed as hypothesis-generating and will require replication in others studies.

In clinical trials as well as in the current clinical practice, quantification of total HDL-C is still the test of choice because its a simple determination, easily performed and affordable,

particularly in the context of such a common condition. Nevertheless levels of HDL-C often do not translate HDL functions.

The presence of biologic active antibodies towards HDL complex might explain why many patients have low levels of HDL-C and why others remain at risk for clinical events, despite having normal lipid levels. The concept that these antibodies may contribute either to the long-term evolution of atherosclerotic lesions or to the triggering of a clinical event can also explain the heterogeneity found in individual patients and large cohorts when it comes to risk factors and clinical outcomes. Furthermore, recent failures in improving clinical outcomes using drugs that increase HDL concentration such as CETP inhibitors may be explained through the eventual presence of these antibodies.

In conclusion, we have established that aHDL antibodies are a newly found family of auto-antibodies, directed towards different structures in the HDL complex and with the capability to interfere with their biologic functions. Confirmation of the high pathogenic potential of these antibodies may lead to the identification of a new biomarker of vascular disease risk, whilst presenting itself as a novel target for a different treatment approach.

The “clinical phase” of this line of research allowed us to identify differences in the pattern of serum aHDL antibodies according to the different manifestations of patients with vascular disease. However these were cross-sectional studies, and we intend to create prospective cohorts of patients in order to confirm whether antibodies towards HDL components will be in fact a risk factor for atherosclerosis progression and vascular events.

Whether some or all of the aHDL antibodies, by causing HDL dysfunction, can induce atheroma progression or plaque instability in such a way as to have clinical significance will be the final, overall aim of this project.

From the basic research point of view, we intent to investigate what are the structural changes (e.g.oxidation, glycation, nitrosilation) or each identified antigen in the HDL complex that are responsible for this acquired humoral response.

Furthermore, we would like to determinate the role of these antibodies in the functional maturation and activation of human dendritic cells and macrophages as they represent an important part of the immune cellular response in atherogenesis.

As one of the main challenges in the development of new HDL raising drugs will be to insure that any change in HDL metabolism triggered by the new drugs also improves the

atheroprotective function of HDL, functional tests for HDL will be critical. The confirmation that aHDL antibodies cause HDL dysfunction may prove to be an indirect way of testing HDL functionality.

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