HDL Functionality and Lipoprotein Quality in Diabetes Mellitus

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Contents

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Figures and Tables

List of Abbreviations

ABCA1	ATP-binding cassette (transporter) subfamily A member 1
ABCG1	ATP-binding cassette (transporter) subfamily G member 1
ACCORD	Action to Control Cardiovascular Risk in Diabetes
ACE	Angiotensin converting enzyme
ADA	American Diabetes Association
ADVANCE	Action in Diabetes and Vascular Disease
AGE	Advanced glycation end-product
AIM-HIGH	Atherothrombosis Intervention in Metabolic Syndrome with Low HDL / High Triglycerides and Impact on Global Health Outcomes
ANOVA	Analysis of variance
Apo	Apolipoprotein
ARBITER 6-HALTS	Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol 6 – HDL and LDL Treatment Strategies in Atherosclerosis
ASTEROID	A Study To Evaluate the Effect of Rosuvastatin on Regression of Coronary Atherosclerosis
BCA	Bicinchoninic acid
BHT	Butylated hydroxytoluene
BP	Blood pressure
BSA	Bovine Serum Albumin
CAC	Coronary artery calcification
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CARDS	Collaborative Atorvastatin Diabetes Study
CCM	Corneal confocal microscopy
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
$CHCl_3$	Chloroform
CHD	Coronary heart disease
CHOD-PAP	Cholesterol oxidase phenol-4-aminoantipyrine peroxidase
CH_3OH / MetOH	Methanol
CI	Confidence Interval
CIMT	Carotid intima media thickness
CML	*N*-ε-carboxymethyl-lysine
CNBD	Corneal nerve branch density
CNFD	Corneal nerve fibre density
CNFL	Corneal nerve fibre length

CRP	C-reactive protein
CT	Computed tomography
Cu^{2+}	Copper
CV	Coefficient of variation
CVD	Cardiovascular disease
DCCT	Diabetes Control and Complications Trial
DEFINE	Determining the Efficacy and tolerability of CETP Inhibition with Anacetrapib
Dox	Deoxy-
DSN	Diabetic sensory neuropathy
ECG	Electrocardiogram
EDTA / Na_2EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FC	Free cholesterol
Fe^{3+}	Iron
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
GlycLDL	Glycated low-density lipoprotein
GOD-PAP	Glucose oxidase phenol 4-aminoantipyrine peroxidase
GPO-PAP	Glycerol-3-phosphate oxidase phenol 4-aminoantipyrine peroxidase
GSPx	Glutathione Peroxidase
HbA1c	Glycated Haemoglobin
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein-cholesterol
HPLC	High performance liquid chromatography
HPS2-THRIVE	Heart protection Study 2-Treatment of HDL to reduce the Incidence of Vascular Events
HRP	Horseradish peroxidase
HSAN1	Hereditary sensory and autonomic neuropathy type 1
hsCRP	High-sensitivity C-reactive protein
H_2O	Water
H_2SO_4	Sulphuric acid
ICAM-1	Intercellular Adhesion Molecule 1
IDL	Intermediate density lipoprotein
ILLUMINATE	Investigation of Lipid Level Management to Understand its Impact in Atherosclerosis Events
IL-6	Interleukin 6
IMPROVE-IT	Improved Reduction of Outcomes: Vytorin Efficacy International Trial

JUPITER	Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin
KOH	Potassium hydroxide
LC	Liquid chromatography
LCAS	Lipoprotein and Coronary Atherosclerosis Study
LCAT	Lecithin cholesterol acyl transferase
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-cholesterol
LOX-1	Lectin-like oxidized low-density lipoprotein receptor 1
Lp(a)	Lipoprotein(a)
LPDS	Lipoprotein deficient serum
LPL	Lipoprotein lipase
LPO	Lipid hydroperoxide
Lp-PLA2	Lipoprotein-associated phospholipase A2
LRG1	Leucine-rich α-2 glycoprotein
$MgCl_2$	Magnesium chloride
MIRACL	Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering
MORGAM	Monica Risk, Genetics, Archiving and Monograph
MPO	Myeloperoxidase
MS	Mass spectrometry
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaN_3	Sodium azide
NDS	Neuropathy disability score
NEFA	Non-esterified fatty acids
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NMDA	*N*-methyl-D-aspartate
NNT	Number needed to treat
NO	Nitric oxide
N_2	Nitrogen
OD	Optical density
OPA	o-phtaldialdehyde
OPD	o-phenylenediamine dihydrochloride
ORIGIN	Outcome Reduction with Initial Glargine Intervention
oxLDL	Oxidized low-density lipoprotein
PANDA	Protection Against Nephropathy in Diabetes with Atorvastatin
PBS	Phosphate-buffered saline
PCSK9	Proprotein convertase subtilisin / kexin type 9

PEG	Polyethylene glycol
PEG-CHE	Polyethylene glycol / cholesterol oxidase
PLTP	Phospholipid transfer protein
PON1	Paraoxonase-1
PPAR-α	Peroxisome proliferator-activated receptor-α
RAGE	Receptors for advanced glycation end products
RCT	Reverse cholesterol transport
REM	Relative electrophoretic mobility
RNA	Ribonucleic acid
SA	Sphinganine
SAA	Serum amyloid A
SCORE	Systematic Coronary Risk Evaluation
SD	Standard deviation
sdLDL	Small-dense low-density lipoprotein
SGLT2	Sodium-glucose cotransporter 2
SO	Sphingosine
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences software
SPT	Serine palmitoyltransferase
SR-B1	Scavenger receptor-B1
STZ	Streptozotocin
S1P	Sphingosine-1-phosphate
TC	Total cholesterol
TETA	Triethylenetetramine
TG	Triglyceride
TGFβ	Transforming growth factor β
TMB	3,3',5,5'-Tetramethylbenzidine
TNB	5-thio-2-nitrobenzoic acid
TNF-α	Tumour necrosis factor alpha
TNT	Treating to New Targets
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
UKPDS	United Kingdom Prospective Diabetes Study
VADT	Veterans Affairs Diabetes Trial
VA-HIT	Veteran Affairs High-Density Lipoprotein Intervention Trial
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low-density lipoprotein
VPT	Vibration perception threshold
3-NT	3-Nitrotyrosine

Abstract

Background & Aims

The 'high-density lipoprotein (HDL) hypothesis', that therapeutic interventions directed at raising HDL cholesterol might translate into improved cardiovascular outcomes, has been confounded by recent reports from genetic and pharmacological studies. HDL functionality may be more important than cholesterol cargo. HDL cholesterol levels are normal or even high in Type 1 Diabetes (T1DM) but do not seem to protect against atherosclerosis as might be expected; this thesis aims to offer new insight into HDL functionality through examination of these patients. This thesis also aims to improve understanding of the qualitative changes in lipoproteins associated with diabetes and increased cardiovascular morbidity, with emphasis on atherogenic modifications of apolipoprotein B and sphingolipids, and consideration of the relationship between these changes, novel and established biomarkers, and macrovascular and microvascular diabetic complications.

Materials & Methods

Patients with Type 1 (n = 91) and Type 2 (n = 40) Diabetes Mellitus and healthy volunteers (n = 104) attended for fasting blood tests, urinalysis, and examination including cardiac computed tomography, carotid doppler studies and assessments of nerve function. *In vitro* studies of lipoprotein modification used pooled human plasma.

Results

Lipoprotein glycation represents an atherogenic modification. *In vitro* glycation occurs more readily in the presence of physiological concentrations of copper. HDL and copper-selective chelation with triethylenetetramine prevents glycation. Glycated apolipoprotein B, oxidized LDL and small-dense LDL levels were significantly higher in T1DM; HDL cholesterol levels were also significantly higher, but with altered apolipoprotein distribution, and significantly lower cholesterol efflux capacity and PON1 activity than in healthy controls. Significant changes were also observed in cystatin C, advanced glycation end-products, leucine-rich α-2-glycoprotein, lipoprotein-associated phospholipase A2, a variety of inflammatory markers, and sphingolipid and ceramide profiles.

Discussion

Cardiovascular disease is the leading cause of death and disability in diabetes. Patients with diabetes show qualitative and kinetic lipoprotein abnormalities, and any cardiovascular benefit associated with intensive glucose lowering may be related to effects on lipoprotein metabolism rather than directly through altered glycaemia. The apparently relatively undisturbed lipid profile observed in many patients with diabetes hides major atherogenic changes and altered HDL functionality, which may be at least partially responsible for the

persistent increased risk of cardiovascular disease in patients with diabetes. HDL-based therapy remains a largely unfulfilled promise, but there may be a role for copper-selective chelation and more aggressive low-density lipoprotein lowering in the reduction of diabetic complications.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university of institute of learning

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Rationale for submitting this thesis in an alternative format

Writing and publishing articles throughout my studies has been helpful in gaining feedback from academics outside the University of Manchester, and has offered opportunities for collaboration. This thesis contains selected publications from my period of registration, and the format has been constructed to include reviews of the relevant literature, explanations of the research methodology employed to reflect my training, and original research papers. This format will increase the impact of this work and has already facilitated dissemination and publication.

Acknowledgement

I keep six honest serving men
(They taught me all I knew);
Their names are What and Why and When
And How and Where and Who

Rudyard Kipling

This thesis is dedicated to my parents, my understanding wife, and Michael and Eleanor. I would like to express my gratitude to my supervisors, Handrean Soran and Rayaz Malik for their mentorship, and all those who have supported me on this journey, particularly Yifen Liu, Garth Cooper, Arnold von Eckardstein, and Thorsten Hornemann.

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Introduction

The Framingham Heart Study identified high-density lipoprotein (HDL) cholesterol to be an independent risk factor for cardiovascular disease [1, 2]. The Emerging Risk Factors and Prospective Studies Collaborations showed that the inverse relationship between HDL cholesterol and cardiovascular risk persists even when low-density lipoprotein (LDL) cholesterol is less than 2.6 mmol/l [3-6], suggesting significant residual cardiovascular risk after LDL cholesterol lowering [7]. Importantly, in the general population HDL cholesterol levels greater than 1.7 mmol/l appear to offer protection against cardiovascular disease, even against LDL cholesterol levels greater than 5.7 mmol/l [8].

Recent Mendelian randomization studies and clinical trials have cast doubt on the viability of the 'HDL hypothesis', that therapeutic interventions directed at raising HDL cholesterol might translate into improved cardiovascular outcomes [9]. HDL cholesterol levels predict neither HDL functionality or composition [10], and high levels do not always protect against cardiovascular disease [11]. It increasingly appears that any therapeutic agent targeting HDL will be required to improve its functionality rather than simply increasing its cholesterol cargo [12]. HDL function can be measured *in vitro* [13]. Determining HDL function may improve the identification of people at increased risk of cardiovascular disease despite unremarkable lipid profile measurements.

In this thesis I consider the potential clinical utility of HDL functionality assessments, examining how they might be used to further define cardiovascular risk, particularly in patients with low HDL cholesterol and patients with diabetes mellitus.

Diabetes mellitus is associated with a considerably increased risk of premature atherosclerosis [14, 15]. The mechanisms responsible for this excess risk remain poorly understood. In type 1 diabetes HDL cholesterol levels are often normal or even high unless glycaemic control is poor or nephropathy has developed [16]; it seems reasonable to conclude that this HDL cholesterol may be dysfunctional, and patients with type 1 diabetes therefore present an excellent opportunity to examine HDL functionality. This thesis considers the contribution of HDL functionality to both macrovascular and microvascular diabetic complications.

For now lowering LDL cholesterol remains the primary focus in lipid modification for the prevention and treatment of atherosclerosis [17], but the relatively unremarkable basic lipid profile of many patients with diabetes may hide major atherogenic changes [18-20]. The number needed to treat (NNT) to prevent one cardiovascular event is lower in patients with diabetes compared to those without diabetes but apparently similar lipid profiles [21].

In this thesis I consider the qualitative changes in lipoproteins that might account for the increased cardiovascular risk observed in patients with diabetes, including factors contributing to the atherogenic modification of LDL cholesterol required for foam cell formation. I examine the role of glucose (and species derived from glucose) in inducing these atherogenic modifications, and explore the reasons underlying the relative ease of lipoprotein glycation *in vivo*. This work encompasses the potential contribution of transition metal ions to *in vitro* lipoprotein glycation, and whether any effect due to copper can be attenuated by the copper chelator triethylenetetramine. Investigation of factors affecting atherogenic modifications of LDL returns us to assessments of HDL functionality and the ability of HDL cholesterol to prevent these modifications.

This thesis also examines selected biomarkers for cardiovascular disease in patients with type 1 diabetes and their association with subclinical atherosclerosis assessed through cardiac computed tomography and carotid doppler studies.

Sphingolipids are a heterogenous class of lipids, including ceramides, which have also been implicated in the development of atherosclerosis and diabetes. Here I extend previous work to explore the potential of these bioactive lipids in the prediction of complications in both type 1 and type 2 diabetes mellitus.

Methodology

Patient Recruitment

I recruited patients with Type 1 and Type 2 Diabetes Mellitus through Central Manchester University Hospitals NHS Foundation Trust, Lancashire Teaching Hospitals NHS Trust, the University Hospital of South Manchester, and the Diabetes Research Network 'Consent for Approach' Database (Help Diabeates®). The study was explained to potential participants and a patient information sheet provided. Those who decided to take part in the study were recruited through a process of informed consent. The study was performed according to the Declaration of Helsinki and was approved by the National Research Ethics Service.

The age range for inclusion in the study was 18 – 75 years. Patients with clinical or electrocardiogram (ECG) evidence of coronary heart disease were excluded. Healthy controls were approached through the University of Manchester, Central Manchester University Hospitals NHS Foundation Trust and Barlow Medical Centre. Healthy controls with any history suggestive of coronary heart disease, vascular insufficiency or diabetes, or using any lipid-lowering drugs or omega fatty acid supplements were excluded.

Patients with Type 1 Diabetes attended the Wellcome Trust Clinical Research Facility at Central Manchester University Hospitals for one visit, where a brief medical questionnaire was administered and examination (including height, weight, waist circumference, blood pressure and ECG) performed. Fasting blood and urine samples were taken. These patients also had assessments of carotid intima media thickness (cIMT) and atheroma burden by carotid doppler studies (thanks to Mr. Mike Smillie and Mrs. Sujamole Subin) and assessment of nerve function and structure by corneal confocal microscopy (CCM) (performed by myself, Dr. Maryam Ferdousi and Dr. Mitra Tavakoli). I also utilised non-invasive tests of nerve function including assessment of neurological disability, nerve electrophysiological testing, quantitative sensory testing and validated questionnaires assessing symptoms of neuropathy.

A second cohort of patients with Type 1 Diabetes was recruited through the Department of Diabetes and Endocrinology at the Bradford Institute for Health Research (thanks to Dr. James Hobkirk). These patients also attended for one visit, where a brief medical questionnaire was administered and examination (including height, weight, waist circumference, blood pressure and ECG) performed. Fasting blood and urine samples were taken. This cohort also had retinal screening and Coronary Artery Calcium Scoring by cardiac computed tomography (CT).

Patients with Type 2 Diabetes attended the Manchester Diabetes Centre for one visit, where a brief medical questionnaire was administered and examination (including height,

weight, waist circumference, blood pressure and ECG) administered. A fasting blood sample was taken and urinalysis performed. Patients with Type 2 Diabetes also had non-invasive tests for assessment of evidence of neuropathy (thanks to Dr. Shazli Azmi).

Healthy volunteers attended the Wellcome Trust Clinical Research Facility or Cardiovascular Trials Unit at Central Manchester University Hospitals for one visit, where I administered a brief medical questionnaire and examination (including height, weight, waist circumference and blood pressure). A fasting blood sample was taken and urinalysis performed.

Glycated Haemoglobin (HbA1c)

Principle

HbA1c is measured primarily to estimate glycaemic control over the previous 3 months, and is formed by non-enzymatic glycation following exposure of haemoglobin to glucose.

Procedure

HbA1c was measured by HPLC using a VARIANT II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hemel Hempstead, UK) in the Department of Clinical Biochemistry at Central Manchester University Hospitals.

Laboratory analyses utilising Cobas Mira and Randox auto-analysers were performed with thanks to Dr. Yifen Liu in the Core Technology Facility at the University of Manchester.

Apolipoprotein A-I (Apo AI)

Principle

Apo AI is measured using an immunoturbidimetric assay adapted for the Cobas-Mira auto-analyzer. The immune complex formed is measured by turbidimetry with the signal generated correlating directly with the concentration of apo AI in the sample. Precision for this immunoturbidimetric immunoassay was intra- and inter-assay CV of 2.2 and 4.8% respectively.

Procedure

1. Add 7μl of sample to 60μl H_2O, 200μl of PBS Polymer solution, 23.3μl of purified immunoglobulins from rabbit antiserum (apo AI from human HDL immunogen) and 46.7 μl PBS
2. Measure generated signal at 340 nm after 10 and 15 minutes using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

Apolipoprotein A-II (Apo AII)

Principle

Apo AII is measured by immunoturbidimetric immunoassay.

Procedure

1. Add 5 μl of sample to 20 μl H_2O, 200μl of PBS Polymer solution, 150 μl of purified immunoglobulins from rabbit antiserum (apo E from human HDL immunogen) and 50 μl PBS
2. Measure generated signal at 600 nm after 10 and 15 minutes using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

Apolipoprotein B (Apo B)

Principle

Apo B is measured immunoturbidimetrically. The immune complex formed is measured by turbidimetry where the signal generated correlates directly with the concentration of apo B in the sample. This immunoassay has intra- and inter-assay CV of 2.2 and 2.6% respectively.

Procedure

1. Add 13 μl of sample to 30 μl of H_2O, 200 μl of PBS polymer solution, 16.7 μl of anti-human apo B antibody and 53.3 μl of PBS
2. Measure generated signal at 340 nm using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

Total Cholesterol

Principle

After enzymatic hydrolysis by cholesterol esterase, cholesterol is oxidized by cholesterol oxidase. The released hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to form quinoneimine. The increase in absorption at 500 nm correlates with cholesterol concentration. This enzymatic endpoint CHOD-PAP method has intra- and inter-assay coefficients of variation (CV) of 2.7 and 3.4% respectively.

Procedure

1. Add 3 μl to 20 μl H_2O and 250 μl reagent
2. Measure increase in absorbance at 500 nm using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

C-reactive protein (CRP)

Principle

CRP is an acute-phase protein of hepatic origin that increases in response to interleukin-6 secretion. It can be measured by immunoturbidimetric assay with intra- and inter-assay CV of 1.2 and 2.9% respectively.

Procedure

1. Add 2.5 μl sample to reaction buffer with CRP immunoparticles
2. Measure generated signal at 340 nm after 10 and 15 minutes using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

Creatinine

Principle

Creatinine is derived from creatine and creatine phosphate and is excreted in the urine at a constant rate. Since its rate of production is also constant, elevated plasma creatinine suggests reduced excretion and thus renal impairment. Creatinine in alkaline solution reacts with picrate to form a coloured complex. The assay has intra- and inter-assay CV of 4.0 and 5.0% respectively for serum, and 2.1 and 3.0% respectively for urine.

Procedure

1. Add sample to sodium hydroxide (0.2 mol/l) and picric acid (25 mmol/l)
2. Measure generated signal using Randox auto-analyser (Randox, Co. Antrim, UK)

Cystatin C

Principle

Cystatin C is freely filtered by the glomerular basement membrane, making circulating levels a good indicator of glomerular function. The immunoturbidimetric method has intra- and inter-assay CV of 2.6 and 4.4% respectively.

Procedure

1. Add sample to Cystatin Assay Buffer and Cystatin Antibody Reagent
2. Measure generated signal at 570 nm after 10 and 15 minutes using Randox Daytona auto-analyser (Randox, Co. Antrim, UK)

Glucose

Principle

Glucose oxidase converts glucose into gluconic acid generating hydrogen peroxide, which reacts with phenol and 4-aminoantipyrine in the presence of peroxidase. The increase in absorbance at 505 nm correlates with the glucose concentration. This GOD-PAP method is linear for glucose concentrations up to 22.2 mmol/l.

Procedure

1. Add 50 μl of sample to 200 μl peroxidase and 50 μl glucose
2. Incubate at room temperature for 20 minutes
3. Measure increase in absorbance at 510 nm using Cobas Mira auto-analyser (ABX Horiba-UK) and calculate concentration

High-density lipoprotein (HDL) cholesterol

Principle

Polyethylene glycol (PEG) modified cholesterol esterase and cholesterol oxidase enzymes show selective catalytic activities towards lipoprotein fractions. Combination of PEG-modified enzymes with α-cyclodextrin sulphate allows selective determination of HDL-C in serum. HDL-cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by PEG-modified cholesterol esterase. In the presence of oxygen cholesterol is oxidized by cholesterol oxidase and generated hydrogen peroxide reacts with 4-aminoantipyrine and N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline. The increase in absorbance at 600 nm correlates with the HDL-cholesterol concentration. This PEG-CHE method has intra- and inter-assay CV of 1.2 and 0.9% respectively.

Procedure

1. Add 3 μl of sample to 50 μl H_2O, 250 μl of reagent 1 (*N*,*N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonphonic acid, *N*-(2-hydroxy-3-Sulfopropyl)-3,5-dimethoxyaniline, sodium salt, cholesterol esterase, cholesterol oxidase, catalase and ascorbate oxidase), 83 μl of reagent 2 (*N*,*N*-Bis(2-hydroxyethyly)-2-aminoethanesulphonic acid, 4-aminoantipyrine, horse radish peroxidase, sodium azide and surfactants) and 12 μl H_2O
2. Measure increase in absorbance at 600 nm using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

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

Principle

CRP levels within the normal range have been associated with cardiovascular risk. This immunoturbidimetric method has intra- and inter-assay CV of 2.6 and 4.4% respectively.

Procedure

1. Add sample to Assay Buffer (Glycine, sodium chloride, sodium EDTA disodium salt dihydrate and BSA) and Antibody-latex reagent (Latex particles coated with antibody to CRP)
2. Measure generated signal at 570 nm after 10 and 15 minutes using Randox Daytona auto-analyser (Randox, Co. Antrim, UK)

Microalbumin

Principle

Albumin is one of the major plasma proteins and in normal circumstances does not cross the glomerular basement membrane. The absorbance of the turbid solution formed by addition of undiluted sample to buffer containing antibody specific for human albumin is proportional to the concentration of albumin in the sample urine.

Procedure

1. Add sample to Assay Buffer (Polyethylene glycol, Tris / HCl buffer, sodium chloride and sodium azide) and Antibody Reagent (Anti-human albumin, Tris / HCl buffer, sodium chloride and sodium azide)
2. Measure generated signal at 340 nm after 10 and 15 minutes using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

Small-dense LDL (sdLDL)

Principle

Chylomicrons, VLDL, IDL, buoyant LDL and HDL are decomposed by surfactant and sphingomyelinase before another surfactant releases cholesterol from sdLDL which is subjected to hydrolysis by cholesterol esterase and oxidation by cholesterol oxidase; produced hydrogen peroxides generate a purple / red colour in the presence of peroxidase. Intra-assay CV for this assay was 2.5%.

Procedure

1. Add 6 μl sample to 300 μl Reagent 1 (Good's buffer, Cholesterol esterase, cholesterol oxidase, sphingomyelinase, catalase, *N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline and BSA)
2. Incubate for 3 – 5 minutes and read absorbance at 600 nm

3. Add 100 μl Reagent 2 (Good's buffer, Horseradish Peroxidase, 4-aminoantipyrine and sodium azide)
4. Incubate for 5 minutes and read absorbance at 600 nm using Randox Daytona (Randox, Co. Antrim, UK)

Triglyceride

Principle

Oxidation by glycerol-3-phosphate oxidase releases hydrogen peroxide, which generates quinoneimine from 4-aminoantipyrine and phenol in the presence of peroxidase. The increase in absorbance at 500 nm correlates with the triglyceride concentration. This GPO-PAP method has intra- and inter-assay CV of 3.3 and 3.5% respectively.

Procedure

1. Add 3 μl of sample to 10 μl H_2O and 290 μl reagent
2. Measure increase in absorbance at 500 nm using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK)

Uric Acid

Principle

Uric acid is derived from the breakdown of purine nucleotides. Uricase cleaves uric acid to form allantoin and hydrogen peroxide. In the presence of peroxidase, 4-aminophenazone is oxidized by hydrogen peroxide to a quinone-diimine dye, which can be measured by Cobas (Roche Diagnostics, Indianapolis, IN, USA) with intra- and inter-assay CV of 0.6 and 1.3% respectively.

Procedure

1. Add 3 μl sample to 72 μl Phosphate Buffer / *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline / fatty alcohol polyglycol ether / ascorbate oxidase with 25 μl H_2O and 14 μl Phosphate Buffer / potassium hexacyanoferrate / 4-aminophenazone / uricase / peroxidase with 25 μl H_2O
2. Read increase in absorbance at 546 nm and calculate concentration

Enzyme-linked immunosorbent assays were performed with the assistance of Dr. Yifen Liu and Mrs. Tarza Siahmansur in the Core Technology Facility at the University of Manchester.

High-sensitivity Apolipoprotein B

Principle

This apo B ELISA technique is based on the antibody sandwich principle.

Procedure

1. Add 100 μl Capture antibody (6 μl goat anti-human apolipoprotein antibody (Abcam, Cambridge, UK) in 12 ml PBS buffer) to each well and cover with plate seal
2. Incubate overnight at 4°C
3. Wash plate 3 times using Wash buffer (0.05% v/v Tween-20 in PBS)
4. Add 300 μl Reagent diluent (1% BSA in PBS) to each well and incubate at room temperature for 60 minutes
5. Wash plate 3 times using Wash buffer
6. Add 100 μl Reagent diluent with 0.1% Tween-20, 100 μl apo B standards (Sigma-Aldrich, Dorset, UK), or 100 μl diluted serum samples to plate wells; cover plate and incubate at room temperature for 60 minutes
7. Wash plate 3 times using Wash buffer
8. Add 100 μl Detection antibody (lyophilized HRP-labelled goat polyclonal anti-human apolipoprotein B (Abcam, Cambridge, UK) with Reagent diluent and Tween); cover plate and incubate at room temperature for 60 minutes
9. Wash plate 3 times using Wash buffer
10. Add 100 μl OPD (Sigma-Aldrich, Dorset, UK) to each well at 20 second intervals and incubate at room temperature for 5 – 10 minutes
11. Add 50 μl stop solution (1.25 M sulphuric acid) to each well at 20 second intervals
12. Read optical density at 492 nm and calculate results

Apolipoprotein B48 (Apo B48)

Principle

This ELISA (MyBioSource.com, San Diego, CA, USA) employs a competitive inhibition reaction with intra- and inter-assay CV of 7.5 and 9.5% respectively.

Procedure

1. Add 50 μl standard and sample to each well (pre-coated with apo B48)
2. Add 50 μl HRP-conjugate to each well; cover and incubate for 30 minutes at 37°C
3. Aspirate each well and wash 5 times with 200 μl wash buffer
4. Add 90 μl TMB Substrate to each well and incubate for 20 minutes at 37°C in the dark
5. Add 50 μl Stop Solution to each well and gently mix
6. Immediately determine the optical density at 450 nm
7. Calculate concentration from standard curve

Apolipoprotein C-III (Apo CIII)

Principle

This ELISA (Sigma-Aldrich, St. Louis, MO, USA) has intra- and inter-assay CV of 9.5 and 11.5% respectively.

Procedure

1. Add 100 μl standard or sample to Human Apo CIII Antibody-coated ELISA plate; cover and incubate overnight at 4°C with gentle shaking
2. Wash 4 times with 300 μl Wash solution
3. Add 100 μl Biotinylated Human Apo CIII Detection Antibody to each well; cover and incubate for 1h at room temperature with gentle shaking
4. Wash 4 times with 300 μl Wash solution
5. Add 100 μl HRP-Streptavidin solution to each well; cover and incubate for 45 minutes at room temperature with gentle shaking
6. Wash 4 times with 300 μl Wash solution
7. Add 100 μl Colorimetric TMB Substrate reagent to each well; cover and incubate for 30 minutes at room temperature in the dark with gentle shaking
8. Add 50 μl Stop Solution to each well and immediately read absorbance at 450 nm
9. Calculate concentration from standard curve

High-Sensitivity Apolipoprotein E (Apo E)

Principle

In this in-house sandwich ELISA a polyclonal Apo E antibody is coated onto microplate wells.

Procedure

1. Add 100 μl dilute polyclonal goat anti-human Apo E antibody (Academy Bio-Medical Company, Houston, TX, USA) to microplate wells and shake gently overnight at 4°C.
2. Wash with 0.01% (v/v) Tween-20 PBS Wash buffer
3. Add 150 μl 5% BSA in PBS Blocking buffer and shake gently for 2h at room temperature
4. Wash 4 times with Wash buffer
5. Add 100 μl standard and samples; shake gently overnight at 4°C
6. Wash 4 times with Wash buffer
7. Add 100 μl HRP-conjugated goat anti-human Apo E (Academy Bio-Medical Company, Houston, TX, USA); shake gently at room temperature for 2h
8. Wash 4 times with Wash buffer
9. Add 100 μl TMB Enzyme substrate; shake gently for 20 minutes
10. Add 50 μl 2M H_2SO_4

11. Read absorbance at 453 nm and calculate concentration from standard curve

Apolipoprotein M (Apo M)

Principle

The Apo M ELISA (Holzel Diagnostika Handels GmbH, Cologne, Germany) applies a quantitative sandwich immunoassay.

Procedure

1. Add 50 μl standards or samples to appropriate well of Apo M monoclonal antibody pre-coated microtitre plate
2. Add 100 μl of conjugate to each well and mix well; cover and incubate for 1h at 37°C
3. Remove incubation mixture by aspirating contents of plate and wash 5 times with wash solution
4. Add 50 μl Substrate A to each well
5. Add 50 μl Substrate B to each well; cover and incubate for 15 minutes at room temperature
6. Add 50 μl Stop solution to each well and mix well
7. Read Optical Density at 450 nm using microtitre plate and determine concentration using standard curve

N-ε-carboxymethyl-lysine (CML)

Principle

N-ε-carboxymethyl-lysine (CML) was measured by sandwich ELISA (MyBioSource Inc., USA), with intra- and inter-assay coefficients of variation of 7.5 and 9.5% respectively.

Procedure

1. Add 100 μl of standard and sample to wells pre-coated with antibody specific for CML; cover and incubate for 2h at 37°C
2. Remove liquid and add Biotin-antibody to each well without washing; cover and incubate for 1h at 37°C
3. Aspirate and wash each well 3 times with 200 μl Wash buffer
4. Add 100 μl HRP-avidin to each well; cover and incubate for 1h at 37°C
5. Aspirate and wash each well 5 times with 200 μl Wash buffer
6. Add 90 μl TMB substrate to each well and incubate for 15 – 30 minutes at 37°C in the dark
7. Add 50 μl Stop Solution to each well and gently mix

8. Immediately determine optical density using a microplate reader set to 450 nm and calculate the concentration using the standard curve

Glycated LDL

Principle

Glycacor (Exocell, Philadelphia, PA, USA) is an indirect competitive ELISA for the determination of glycated LDL in plasma. The mouse monoclonal antibody ES12 recognizes a specific epitope on glycated apolipoprotein B. The assay has intra- and inter-assay CV of 3.5 and 14.9% respectively.

Procedure

1. Drain wells from Glycacor Assay Plate (microplate pre-coated with standardized preparation of glycated LDL stored in blocking solution), and wash 5 times with Glycacor wash buffer
2. Prepare control and standard wells using LDL diluent and glycated LDL standard
3. Add samples to wells
4. Add 50 μl ES12 anti-glycated LDL to each well; cover and incubate at room temperature for 1h
5. Drain and wash 10 times with Glycacor wash buffer
6. Add 100 μl HRP-conjugate to each well; cover and incubate at room temperature for 1h
7. Drain and wash 10 times with Glycacor wash buffer
8. Add 100 μl colour developer to each well; cover and incubate at room temperature for 10 minutes
9. Add 100 μl colour stopper and determine absorbance at 450 nm; determine concentration of glycated LDL within diluted experimental samples from standard curve

Intercellular Adhesion Molecule 1 (ICAM-1)

Principle

This ELISA (R&D Systems Europe, Abingdon, UK) measures ICAM-1, also known as CD54, a transmembrane protein that is upregulated on endothelial and epithelial cells at sites of inflammation.

Procedure

1. Coat microplate wells with 100 μl diluted Capture Antibody; seal plate and incubate overnight at room temperature
2. Aspirate wells and wash with 400 μl Wash Buffer 3 times

3. Block plates by adding 300 μl Reagent Diluent to each well and incubate at room temperature for 1h
4. Aspirate wells and wash with 400 μl Wash Buffer
5. Add 100 μl sample or standards in Reagent Diluent to each well; cover and incubate for 2h at room temperature
6. Aspirate wells and wash with 400 μl Wash Buffer
7. Add 100 μl Detection Antibody, diluted in Reagent Diluent, to each well; cover and incubate for 2h at room temperature
8. Aspirate wells and wash with 400 μl Wash Buffer
9. Add 100 μl working dilution of Streptavidin-HRP to each well; cover plate and incubate for 20 minutes at room temperature
10. Aspirate wells and wash with 400 μl Wash Buffer
11. Add 100 μl Substrate solution to each well and incubate for 20 minutes at room temperature
12. Add 50 μl Stop Solution to each well and immediately determine the optical density using a microplate reader at 540 nm

Interleukin 6 (IL-6)

Principle

Interleukin-6 acts both as a pro-inflammatory cytokine and anti-inflammatory myokine. Circulating concentrations can be measured by solid phase sandwich ELISA (R&D Systems Europe, Abingdon, UK).

Procedure

1. Dilute Capture Antibody to working concentration in PBS without carrier protein and immediately add 100 μl to each well; seal microplate and incubate overnight at room temperature
2. Aspirate each well and wash 3 times with 400 μl Wash Buffer
3. Add 300 μl Reagent Diluent to each well and incubate at room temperature for 1h
4. Aspirate each well and wash 3 times with 400 μl Wash Buffer
5. Add 100 μl sample or standards in Reagent Diluent to each well; cover and incubate for 2h at room temperature
6. Aspirate each well and wash 3 times with 400 μl Wash Buffer
7. Add 100 μl Detection Antibody diluted in Reagent Diluent to each well; cover and incubate for 2h at room temperature
8. Aspirate each well and wash 3 times with 400 μl Wash Buffer
9. Add 100 μl working dilution of Streptavidin-HRP to each well; cover plate and incubate in the dark for 20 minutes at room temperature

10. Aspirate each well and wash 3 times with 400 μl Wash Buffer
11. Add 100 μl Substrate Solution to each well and incubate in the dark for 20 minutes at room temperature
12. Add 50 μl Stop Solution to each well and gently mix
13. Immediately determine optical density of each well using a microplate reader set to 540 nm

Leucine-rich α-2 glycoprotein (LRG1)

Principle

LRG1 is a pro-angiogenic factor involved in the regulation of the TGFβ signaling. It has been shown to be a significant predictor of endothelial dysfunction and peripheral vascular disease in patients with type 2 diabetes [22]. This ELISA (Immuno-Biological Laboratories, Minneapolis, MN, USA) uses a solid phase sandwich technique and has intra- and inter-assay CV of 3.0 and 4.2% respectively.

Procedure

1. Add 100 μl EIA buffer, sample blank, sample or standard dilutions to wells of microtitre plate pre-coated with Anti-Human LRG Rabbit Immunoglobulin; cover and incubate overnight at 4°C
2. Wash 7 times with wash buffer
3. Add 100 μl labelled antibody (HRP conjugated Anti-Human LRG Rabbit Immunoglobulin) solution to wells; cover and incubate for 30 minutes at 37°C
4. Wash 9 times with wash buffer
5. Add 100 μl TMB solution to each well and incubate for 30 minutes at room temperature in the dark
6. Add 100 μl Stop solution (1N H_2SO_4) to wells and mix
7. Immediately measure absorbance at 450 nm and calculate sample concentration from standard curve

Lipoprotein (a) (Lp(a))

Principle

The Lp(a) ELISA (Mercodia, Uppsala, Sweden) is a solid phase two-site enzyme immunoassay based on the sandwich technique. Apolipoprotein(a) in samples reacts with anti-apolipoprotein(a) antibodies bound to microtitration wells and peroxidase-conjugated anti-apolipoprotein(a) antibodies in solution. Intra- and inter-assay CV were 3.3 and 4.0% respectively.

Procedure

1. Add 25 μl pretreatment solution to 25 μl sample; vortex and incubate for 1h at room temperature
2. Add 5 ml sample buffer and mix
3. Add 25 μl calibrator or 25 μl pretreated sample to anti-Apo(a) wells
4. Add 50 μl enzyme conjugate solution to wells and incubate on a shaker for 1h at room temperature
5. Discard reaction volume and wash 6 times with 350 μl wash buffer solution
6. Add 200 μl Substrate TMB and incubate for 15 minutes
7. Add 50 μl Stop solution and measure absorbance at 450 nm and evaluate

Lipoprotein-associated phospholipase A2 (Lp-PLA2)

Principle

Most PLA2 is associated with LDL, where its activity is an independent risk factor for coronary heart disease [23]. The kit is a sandwich enzyme immunoassay using a microtitre plate pre-coated with an antibody specific to Lp-PLA2 (Uscn Life Science Inc., Buckingham, UK). Intra- and inter-assay CV were 9.5 and 11.5% respectively.

Procedure

1. Add 100 μl standard dilutions / blank / samples to appropriate wells in pre-coated plate; cover and incubate for 2h at 37°C
2. Remove liquid from each well and add 100 μl working solution containing biotin-conjugated antibody specific to Lp-PLA2 to each well; cover and incubate for 1h at 37°C
3. Aspirate solution and wash 3 times with 350 μl Wash solution
4. Remove wash buffer and add 100 μl working solution containing Avidin conjugated to Horseradish Peroxidase to each well; cover and incubate for 30 minutes at 37°C
5. Aspirate solution and wash 5 times with 350 μl Wash solution
6. Remove wash buffer and add 90 μl TMB substrate solution to each well; cover and incubate for 15 – 25 minutes at 37°C in the dark
7. Add 50 μl Sulphuric acid stop solution to each well and mix
8. Immediately run microplate reader at 450 nm and determine concentration by comparing Optical Density of samples to standard curve

MPO Mass

Principle

This in-house ELISA technique is based on the antibody sandwich principle.

Procedure

1. Add 100 μl Capture Antibody (lyophilized rat anti-human MPO (R&D Systems Europe, Abingdon, UK) in PBS) to each well and incubate overnight at room temperature
2. Wash microtitre plate 3 times with Wash buffer (0.05% (v/v) Tween-20 in PBS
3. Add 300 μl Reagent diluent (BSA in PBS and *dd* H_2O) and incubate for 60 minutes at room temperature
4. Wash plate 3 times using Wash buffer
5. Add 100 μl Reagent diluent / MPO standards (R&D Systems Europe, Abingdon, UK) / samples to wells; cover and incubate for 2h at room temperature
6. Wash plate 3 times using Wash buffer
7. Add 100 μl Detection Antibody (lyophilized biotinylated goat anti-human MPO (R&D Systems Europe, Abingdon, UK) with Reagent diluent) to each well; cover and incubate for 60 minutes at room temperature
8. Wash plate 3 times using Wash buffer
9. Add Streptavidin-HRP (R&D Systems Europe, Abingdon, UK) to each well; cover and incubate for 30 minutes in the dark at room temperature
10. Wash plate 3 times using Wash buffer
11. Add 100 μl TMB to each well at 20 second intervals and incubate for 15 – 30 minutes at room temperature
12. Add 50 μl Stop Solution (1M sulphuric acid) to each well at 20 second intervals
13. Read plate on Dynatech MR 7000 Plate reader at 630 nm and calculate concentration from standard curve

Oxidized LDL

Principle

The Oxidized LDL ELISA (Mercodia AB, Uppsala, Sweden) is a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. Precision was calculated as 5.5% intra- and 6.2% inter-assay CV.

Procedure

1. Prepare enzyme conjugate solution, sample and wash buffer solutions, and samples
2. Pipette 25 μl of each calibrator, control and diluted sample into coated plate wells
3. Add 100 μl Assay buffer to each well
4. Incubate on plate shaker for 2h at room temperature
5. Discard reaction volume and add 350 μl wash buffer solution to each well
6. Discard wash solution and tap firmly against absorbent paper to remove excess liquid; add wash buffer and discard 5 times

7. Add 100 μl enzyme conjugate solution to each well
8. Incubate on a plate shaker for 1h at room temperature
9. Discard wash solution and tap firmly against absorbent paper to remove excess liquid; add wash buffer and discard 5 times
10. Add 200 μl 3,3',5,5'-tetramethylbenzidine (TMB) and incubate for 15 minutes at room temperature
11. Add 50 μl Stop solution and place plate on shaker for 5 seconds
12. Read optical density at 450 nm and calculate results
13. Concentration of oxidized LDL is obtained by data reduction of the absorbance for the Calibrators versus the concentration using cubic spline regression; multiply the concentration of the samples with the dilution factor

Proprotein convertase subtilisin / kexin type 9 (PCSK9)

Principle

The primary physiological function of PCSK9 is to mediate the degradation of LDL receptors. This ELISA (R&D Systems Europe, Abingdon, UK) is based on the antibody sandwich principle.

Procedure

1. Add 100 μl Capture Antibody (lyophilized rat anti-human PCSK9 in PBS) to each well and incubate overnight at room temperature
2. Wash plate 3 times using Wash buffer (0.05% (v/v) Tween-20 in PBS)
3. Add 300 μl Reagent diluent (1% BSA in PBS) and incubate for 60 minutes at room temperature
4. Wash plate 3 times using Wash buffer
5. Add 100 μl Reagent diluent / PCSK9 Standards / sample to wells; cover and incubate for 2h at room temperature
6. Wash plate 3 times using Wash buffer
7. Add 100 μl Detection Antibody (lyophilized biotinylated goat anti-human PCSK9 with Reagent diluent); cover plate and incubate for 60 minutes at room temperature
8. Wash plate 3 times using Wash buffer
9. Add 100 μl Streptavidin-HRP to each well; cover and incubate in the dark for 30 minutes at room temperature
10. Wash plate 3 times using Wash buffer
11. Add 100 μl TMB to each well at 20 second intervals and incubate at room temperature for 15 – 30 minutes
12. Add 50 μl Stop Solution (1M sulphuric acid) to each well at 20 second intervals and read absorbance on plate reader at 450 nm

13. Calculate concentration from standard curve

Serum Amyloid A (SAA)

Principle

SAA is a family of acute-phase proteins transported primarily on HDL particles. The human SAA solid-phase sandwich ELISA (ThermoFisher Scientific, Loughborough, UK) has intra- and inter-assay CV of 7.4 and 7.8% respectively.

Procedure

1. Add 100 μl of standards and samples to Human SAA Antibody Coated wells
2. Add 50 μl Human SAA Biotin Conjugate solution to each well; cover plate and incubate for 2h at room temperature
3. Aspirate solution and wash wells 4 times with diluted wash buffer
4. Add 100 μl Streptavidin-HRP to each well; cover plate and incubate for 30 minutes at room temperature
5. Aspirate solution from wells and wash wells 4 times with diluted wash buffer
6. Add 100 μl Stabilized Chromogen to each well; cover plate and incubate for 30 minutes at room temperature in the dark
7. Add 100 μl Stop Solution to each well; read absorbance at 450 nm and calculate concentration from standard curve

Tumour Necrosis Factor alpha (TNF-α)

Principle

TNF-α plays a central role in inflammation, apoptosis and immunity. Quantitative sandwich ELISA (R&D Systems Europe, Abingdon, UK) can be used to determine relative mass values with intra- and inter-assay CV of 5.2 and 7.4% respectively.

Procedure

1. Add 50 μl Assay Diluent to wells coated with mouse monoclonal antibody against TNF-α
2. Add 200 μl Standard, sample or control to each well; cover and incubate for 2h at room temperature
3. Aspirate wells and wash 4 times with 400 μl wash buffer
4. Add 200 μl TNF-α Conjugate to each well; cover and incubate for 2h at room temperature
5. Aspirate wells and wash 4 times with 400 μl wash buffer
6. Add 200 μl Substrate Solution to each well and incubate for 20 minutes at room temperature in the dark

7. Add 50 μl Stop Solution to each well and gently mix
8. Immediately determine optical density at 450 nm and calculate concentration from standard curve

Vascular Cell Adhesion Molecule 1 (VCAM-1)

Principle

This kit (R&D Systems Europe, Abingdon, UK) measures VCAM-1 (or CD106), a transmembrane molecule that mediates the adhesion of immune cells to the vascular endothelium during inflammation.

Procedure

1. Coat microplate wells with 100 μl Mouse Anti-Human VCAM-1 Capture Antibody; seal plate and incubate overnight at room temperature
2. Aspirate each well and wash 3 times with 400 μl Wash Buffer (0.05% Tween-20 in PBS)
3. Block plates by adding 300 μl Reagent diluent (1% BSA in PBS) to each well and incubate at room temperature for 1h
4. Aspirate each well and wash 3 times with 400 μl Wash Buffer
5. Add 100 μl sample or Recombinant Human VCAM-1 Standard in Reagent diluent; cover and incubate for 2h at room temperature
6. Aspirate each well and wash 3 times with 400 μl Wash Buffer
7. Add 100 μl Biotinylated Sheep Anti-Human VCAM-1 Detection Antibody diluted in Reagent diluent to each well; cover and incubate for 2h at room temperature
8. Aspirate each well and wash 3 times with 400 μl Wash Buffer
9. Add 100 μl of working dilution Streptavidin-HRP to each well; cover and incubate for 20 minutes at room temperature
10. Aspirate each well and wash 3 times with 400 μl Wash Buffer
11. Add 100 μl Substrate solution (H_2O_2 and Tetramethylbenzidine) to each well and incubate for 20 minutes at room temperature
12. Add 50 μl Stop solution (2 N H_2SO_4) and immediately determine optical density at 540 nm; calculate concentration from standard curve

3-Nitrotyrosine (3-NT)

Principle

3-NT is thought to be a relatively specific marker of oxidative damage mediated by peroxynitrite. Levels have been reported to be increased in diabetes, and to correlate with postprandial hyperglycaemia [24]. The quantitative sandwich ELISA (MyBioSource Inc. San Diego, CA, USA) has an intra-assay CV of 14.5%.

Procedure

1. Add 50 μl Standard, sample or Sample Diluent to each well
2. Add 100 μl HRP-conjugate reagent to each well; cover and incubate at 37°C for 60 minutes
3. Wash plate 4 times with Wash solution
4. Add 50 μl Chromogen solution A and 50 μl Chromogen solution B to each well; gently mix and incubate in dark for 15 minutes at 37°C
5. Add 50 μl Stop solution to each well and gently mix
6. Immediately read optical density at 450 nm; calculate concentration from standard curve

Preparation of LDL, HDL and subfractions by ultracentrifugation

Principle

Lipoproteins are isolated from fresh EDTA-plasma by a simplified ultracentrifugation method. The density of plasma is raised by the addition of a concentrated solution of Potassium Bromide (heavy density solution). For protection studies, prepare HDL fractions using heavy density solutions that contain Ca^{2+} but no EDTA.

Procedure

1. Adjust density of serum to required density by adding required volume of relevant heavy density solution:

   $$\text{Required volume} = \frac{\text{Required density} - \text{Original density}}{\text{Density of solution to be added} - \text{Required density}} \times \text{Original volume}$$

   Where serum / plasma is at a density of 1.006 g/ml, LDL 1.019 – 1.063 g/ml, and HDL 1.063 – 1.21 g/ml
2. Centrifuge at 40,000 rpm for 24h at 4°C (50.3 Ti rotor) or 100,000 rpm for 3h at 4°C (TLA 120.2 rotor)
3. Discard upper lipid fraction using fine-tipped pipette
4. Reconstitute sample and adjust density to that required using equation above and centrifuge again
5. Remove lipid fraction using fine-tipped pipette and dialyse against 5L PBS or TBS containing 10 mM $CaCl_2$ (for protection studies) for 24h at 4°C
6. Store isolated lipoprotein fraction under nitrogen at 4°C for up to 1 week
7. Measure protein concentration using Pierce kit.

Pierce BCA Protein Assay Kit

Principle

The BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA) is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. The method depends on the reducing properties of proteins. Chelation occurs between two molecules of BCA and one cuprous ion to generate colour.

Procedure

1. Prepare diluted bovine serum albumin (BSA) standards for a working concentration range (20 – 2,000 μg/ml)
2. Pipette 25 μl of each standard or unknown sample replicate into a microplate well
3. Add 200 μl BCA Working Reagent to each well and mix plate on a plate shaker for 30 seconds
4. Cover plate and incubate at 37°C for 30 minutes
5. Cool plate to room temperature and measure the absorbance at 562 nm
6. Prepare standard curve by plotting average blank-corrected 562 nm measurement for each BSA standard against its concentration in μg / ml; use the standard curve to determine the protein concentration of each unknown sample

In vitro Glycation

Principle

Glycated and non-glycated apo B are separated by *m*-aminophenylboronate affinity chromatography where the immobilized gel binds to the *cis-diol* groups on the sugar moiety forming a reversible five-member ring complex. After washing away non-bound molecules the complex can be dissociated and the glycated lipoprotein eluted by the addition of sorbitol.

Procedure

1. Incubate 0.5 ml volumes of LDL (1mg/ml) protein with 0.5 ml sterile TBS (20 mM Tris, 0.9% NaCl, 2 mM $CaCl_2$ 0.01% chloramphenicol) under N_2 in air-tight screw-capped 2 ml polypropylene sample tubes with glucose or δ-gluconolactone in sterile TBS for up to 7 days at 37°C in a Gallenkamp incubator, in the presence and absence of Ascorbic acid, Triethylenetetramine (TETA) or homologous HDL (0.5 mg/ml)
2. Following incubation dialyse overnight at 4°C against TBS to remove excess glucose
3. Add 100 μl of sample to top of *m*-Aminophenylboronate affinity column (following equilibration of column with 10 ml ammonium actetate)
4. Elute non-glycated proteins with 5 ml of ammonium actetate equilibration / wash buffer
5. Elute glycated proteins with 3.9 ml sorbitol elution buffer
6. Regenerate columns for further assays with 10 ml regeneration buffer

Glycated Apo E

Principle

Glycated and non-glycated apo E can be separated by *m*-aminophenylboronate affinity chromatography where the immobilized gel binds to the *cis-diol* groups on the sugar moiety forming a reversible five-member ring complex. After washing away non-bound molecules the complex can be dissociated and the glycated lipoprotein eluted by the addition of sorbitol.

Procedure

1. Add 100 μl of sample to top of *m*-Aminophenylboronate affinity column (following equilibration of column with 10 ml ammonium actetate)
2. Elute non-glycated proteins with 5 ml of ammonium actetate equilibration / wash buffer
3. Elute glycated proteins with 3 ml sorbitol elution buffer
4. Determine glycated, non-glycated and total apo E by high-sensitivity ELISA described above

In vitro Oxidation

Principle

Lipid peroxides are measured spectrophotometrically using iodide containing colourimetric reagent. Lipid hydroperoxides oxidize iodide with the quantity of liberated tri-iodide anion directly proportional to the levels of lipid hydroperoxides in the sample.

Procedure

1. Incubate 0.5 ml volumes of LDL (1mg/ml) protein with 0.5 ml sterile TBS (20 mM Tris, 0.9% NaCl, 2 mM $CaCl_2$ 0.01% chloramphenicol) under N_2 in air-tight screw-capped 2 ml polypropylene sample tubes with glucose or δ-gluconolactone in sterile TBS for up to 7 days at 37°C in a Gallenkamp incubator, in the presence and absence of Ascorbic acid, Triethylenetetramine (TETA) or homologous HDL (0.5 mg/ml)
2. Following incubation dialyse overnight at 4°C against TBS to remove excess glucose
3. Incubate 50 μl of reaction mixture with Cholesterol-Iodine working reagent for 30 minutes at room temperature in the dark
4. Read absorbance at 365 nm
5. Calculate concentration of Lipid peroxides:

$$\text{LPO (nmol/ml)} = \frac{\Delta_{OD}}{2.46 \times 10^4} \times \frac{\text{Volume measured}}{\text{Volume taken}} \times 10^6$$

Agarose gel electrophoresis

Principle

Agarose gel electrophoresis is used to measure the overall change in particle charge after glycation by measuring the relative electrophoretic mobility (REM). The overall charge is determined by the total contribution of arginine, lysine, protonated histidine, and the *N* terminus amine group relative to glutamic, aspartic and *C* terminus carboxyl residues. Glycation leads to modification of positively charged apo B lysine residues, with less positively charged LDL moving further into the gel than native LDL. This method was performed with the assistance of Dr. Yifen Liu.

Procedure

1. Load native and *in vitro* glycated lipoproteins onto 1% agarose gel (30 μl per well) (Wolf laboratories, Pocklington, UK)
2. Electrophorese against barbital buffer pH 8.6 at 70 V, 400 mA for 1h
3. Visualize electrophoresed bands and photograph using Unipro Platinum gel documentation and analysis system (Uvitec, Cambridge, UK)
4. Measure distances travelled by native and glycated lipoproteins
5. Calculate REM as ratio of distance travelled by glycated LDL relative to native LDL

Glycated LDL uptake by cultured macrophages

Principle

Glycated LDL is taken up rapidly by macrophages in culture, and both oxidized and glycated LDL have been demonstrated in atherosclerotic lesions, where they exist at higher concentrations than in the circulation [25]. This method was developed by and performed with the assistance of Dr. Nahla Younis and Dr. Yifen Liu.

Procedure

1. Culture THP-1 cells (ECACC, Salisbury, UK) in suspension in RPMI 1640 medium containing 4 mmol/l glutamine and 1% (v/v) Penicillin / Streptomycin, 1% Non-essential amino acids and 10% (v/v) heat-inactivated Foetal Calf Serum in humidified incubators (5% CO_2, 37^{o}C)
2. Dilute cells to 1 x 10^6 cells / ml in serum free RPMI 1640 media and incubate with phorbol myristate acetate (PMA) under 5% CO_2 at 37^{o}C for 72h to differentiate THP-1 cells into macrophages
3. Collect serum from healthy volunteers and adjust to density 1.25 g / ml using EDTA-free heavy density solution; centrifuge at 34,000 rpm for 22h and 17 minutes at 4^{o}C (Beckman Instruments Ltd, Palo Alto, USA); pool infranatants and dialyse against 0.9%

(v/v) NaCl containing 0.1 mg / ml chloramphenicol to remove all salts; inactivate at 60°C for 30 minutes followed by sterile filtration (0.22 μm) to produce lipoprotein deficient serum (LPDS)

4. Incubate macrophage cultures for 48h in RPMI 1640 media containing 10% LPDS with native LDL and *in vitro* glycated LDL
5. Equilibrate cells overnight in media containing 1 mg / ml BSA after exposure to lipoproteins
6. Lyse cells in ice cold water for 20 minutes at 4°C and scrub wells; centrifuge lysate at 353 x G for 5 minutes to remove cellular debris
7. Determine lipid uptake by THP-1 macrophages by measuring total cholesterol, free cholesterol and cholesteryl esters in loading media, incubation media and cell lysate; total and free cholesterol are measured by the enzymatic photometric cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) method, and cholesteryl esters calculated by subtracting free cholesterol from total cholesterol
8. Wash cultured cells in DPBS for 5 minutes and fix with 4% paraformaldehyde for 20 minutes; wash again in DPBS for 5 minutes and stain with oil red O for 15 minutes; remove excess stain with 60% isopropanol; photograph directly in culture plates with phase contrast microscope fitted with Nikon digital camera

Preparation of Apo B-deplete serum

Principle

HDL isolation by ultracentrifugation is time-consuming and may disrupt HDL particles. Clinical studies increasingly precipitate apo B-containing lipoproteins out of serum to better evaluate HDL functionality.

Procedure

1. Add 400 μl polyethylene glycol (PEG) (Sigma-Aldrich, St. Louis, MO) to 1 ml serum
2. Incubate for 20 minutes at room temperature
3. Centrifuge at 10,000 rpm for 30 minutes and discard pellet

Cholesterol Efflux

Principle

Cholesterol efflux capacity from macrophages is a metric of HDL function. This method was developed by and performed with the assistance of Dr. Yifen Liu.

Procedure

1. Culture 2 ml J774A.1 cells (European Collection of Cell Cultures) in 30 ml RPMI 1640 medium containing 10% Foetal Bovine Serum, 100 IU / ml penicillin and 100 mg / ml streptomycin at 37°C with 5% carbon dioxide for 2 – 4 days
2. Pellet cells by centrifugation (1500 rpm for 5 minutes at 4°C) and wash with Hanks' balanced solution (20 ml)
3. Repeat centrifugation and mix with 5 – 10 ml culture medium
4. Plate at final concentration of 5 x 10^5 cells / ml in 12-well tissue culture plates (1 ml / well) for 1 – 2 days
5. Treat 1 ml serum with 400 μl 20% polyethylene glycol solution to precipitate apo B-containing lipoproteins
6. Remove precipitate after 20 minutes by centrifugation (10,000 rpm at 4°C for 30 minutes)
7. Wash plated cells and incubate with 0.2 μCi of radiolabelled ^{3}H-cholesterol / ml in RPMI 1640 medium with 0.2% Bovine Serum Albumin at 37°C and 5% carbon dioxide for 1 day
8. Upregulate ABCA1 by using medium containing 0.3 mM 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt for 4h
9. Wash cells and add to 2.8% apo B-depleted serum
10. Collect cell media after 4h and wash cells with PBS
11. Dissolve in 0.5 ml 0.2 NaOH for determination of radioactivity

$$\text{Cholesterol efflux (\%)} = \frac{\text{Radioactivity in medium}}{\text{Radioactivity in cell + radioactivity in medium}} \times 100$$

Measurements of enzymatic zctivity using radiological methods were performed with the assistance of Dr. Yifen Liu in the Core Technology Facility at the University of Manchester.

Cholesteryl Ester Transfer Protein (CETP) Activity

Principle

CETP transfers cholesteryl esters from HDL to VLDL / LDL in both radioactive and plasma cholesteryl esters at 37°C.

Procedure

1. Add 50 μl [^{3}H] cholesterol albumin emulsion to 0.5 ml EDTA plasma and vortex at 4°C for 1h
2. Incubate at 37°C for 3h
3. Centrifuge at 2,000 rpm for 5 minutes
4. Aliquot 300 μl supernatant and add 60 μl 0.5 M $MgCl_2$ / 4% Phosphotungstic acid
5. Add 3 ml wash solution (100 ml 0.9% saline with 100 μl $MgCl_2$ and 100 μl phosphotungstate) and vortex
6. Spin at 2,000 rpm for 20 minutes at 4°C

7. Aliquot 100 μl supernatant to HDL inserts and discard rest of supernatant
8. Wash pellet with 3 ml wash solution and vortex
9. Spin at 3,000 rpm for 15 minutes at 4°C and decant supernatant
10. Add 250 μl 2:1 Chloroform : Methanol to precipitate and vortex
11. Leave at 4°C for 1h then add 50 μl 0.9% saline and vortex
12. Spin at 2000 rpm for 10 minutes at 4°C and spot organic phase onto thin-layer chromatography plate
13. Develop in 101 ml hexane : diethyl ether : glacial acetic acid (80 : 20 : 1)
14. Add 400 μl ethanol
15. Add 2 ml Scintillation cocktail and count radioactivity

CETP activity = $\frac{\text{Free cholesterol concentration}}{\text{incubation time (3h)}} \times \frac{\text{dpmCE}}{\text{dpmInc}} \times \frac{(\text{dpmInc} - 5.59\text{dpmHDL})}{(\text{dpmFC} + \text{dpmCE})}$

Where: dpmCE is radioactivity in cholesteryl ester in phosphotungstate precipitate
dpmInc is radioactivity in 50 μl aliquot of incubation system
dpmHDL is radioactivity in 100 μl $MgCl_2$ sodium Phosphotungstate supernatant
dpmFC is radioactivity in free cholesterol in precipitate

Lecithin Cholesterol Acyl Transferase (LCAT) Activity

Principle

LCAT is responsible for the esterification of cholesterol and is located on HDL.

Procedure

1. Add 50 μl [^{3}H] cholesterol albumin emulsion to 0.5 ml EDTA plasma and vortex at 4°C for 1h
2. Incubate at 37°C for 3h
3. Centrifuge at 2,000 rpm for 5 minutes
4. Aliquot 150 μl and add 3 ml $CHCl_3$ / CH_3OH
5. Add 600 μl 0.9% saline at 4°C and vortex
6. Spin at 2,000 rpm for 10 minutes at 4°C
7. Draw off inorganic phase and middle protein layer
8. Evaporate residual organic phase to dryness at 100°C
9. Re-dissolve residue in 250 μl $CHCl_3$ / CH_3OH
10. Leave at 4°C for 1h then spot onto TLC plate
11. Develop in 101 ml hexane : diethyl ether : glacial acetic acid (80 : 20 : 1)
12. Add 400 μl ethanol
13. Add 2 ml Scintillation cocktail and count radioactivity

LCAT activity = $\frac{\text{plasma free cholesterol concentration}}{\text{incubation time (3h)}} \times \frac{(\text{dpmCE})}{(\text{dpmFC} + \text{dpmCE})}$.

Where: dpmCE is radioactivity in cholesteryl ester in lipid extract
dpmFC is radioactivity in free cholesterol in lipid extract

Myeloperoxidase (MPO) Activity

Principle

MPO catalyzes the formation of hypochlorous acid, which reacts with taurine to form taurine chloroamine. In this colourimetric activity assay kit (Sigma-Aldrich, St. Louis, MO, USA) 1 unit of MPO activity is defined as the amount of enzyme that hydrolyzes the substrate and generates taurine chloroamine to consume 1 μmol of TNB per minute at room temperature.

Procedure

1. Add 50 μl Reaction Mix (MPO Assay Buffer and H_2O or MPO Substrate) to positive control, sample and sample blank wells and mix well using a horizontal shaker in the dark at room temperature
2. Read assay at 3 time points (30, 60 and 120 minutes) to ensure values are in linear range of standard curve; at each time point add 2 μl Stop Mix and mix well
3. Incubate for 10 minutes to stop reaction then add 50 μl TNB Reagent / Standard to each well
4. At final time point add serial dilutions of TNB Reagent / Standard to Assay Buffer-containing standard wells and incubate for 10 minutes
5. Measure absorbance at 412 nm and calculate change in absorbance due to consumption of TNB Reagent / Standard by MPO-generated taurine chloramine from linear range of standard curve

Paraoxonase-1 (PON1) Activity

Principle

Serum PON-1 activity is determined by a semi-automated micro-titre plate method using paraoxon (*O,O*-Diethyl *O*-(4-nitrophenyl)phosphate) as a substrate. The rate of generation of *p*-nitrophenol is determined at 25°C with the use of a continuously recording spectrophotometer at 405 nm.

Procedure

1. Add 10 μl of each sample to 200 μl paraoxon stock solution (18.18 μl diethyl *p*-nitrophenyl phosphate with 20 ml 100 mM Tris / 2 mM $CaCl_2$ buffer)
2. Record change in OD at 405 nm with continuous spectrophotometry using multiskan multisoft plate reader (Labsystems, Hampshire, UK) and calculate activity
   PON1 activity (nmol / ml / min) = OD / min x 1390.7 x 1.714

I performed sphingolipid, deoxysphingolipid, and ceramide analysis in the Institute for Clinical Chemistry at the University Hospital Zurich with thanks to Prof. Thorsten Hornemann and Ms. Regula Steiner.

Lipid Extraction and Acid / Base Hydrolysis for Sphingolipids and Deoxy-sphingolipids

Principle

Sphingolipids are a heterogeneous class of lipids derived from the aliphatic amino-alcohol sphingosine. Acid hydrolysis releases sphingoid base backbones to enable quantification.

Procedure

1. Take 100 µl plasma into a 2 ml polypropylene tube
2. Add 500 µl MetOH (including 0.2 µl D7-SA + 0.2 µl D7-SO) and vortex
3. Shake at 37°C for 1h
4. Centrifuge at full speed for 5 minutes and transfer 500 µl of the supernatant into a new 2 ml tube (discard pellet)
5. Add 75 µl HCl (32%) and vortex
6. Keep at 65°C for 12 – 15h then add 100 µl 10M KOH (56.11g KOH in 100 ml H_2O) and vortex
7. Add 125 µl $CHCl_3$ and vortex
8. Add 500 µl $CHCl_3$ and vortex
9. Add 100 µl 2N Ammonia (15 ml 25% Ammonia Solution + 85 ml H_2O)
10. Add 0.5 ml Alkaline water (500 µl Ammonia solution in 250 ml H_2O + 20 µl 2.5% Bromphenol blue solution) and centrifuge at full speed for 5 minutes
11. Remove upper phase and wash twice with 1 ml of alkaline water
12. Dry under N_2 (samples can be stored at -20°C)

Lipid Extraction and Base Hydrolysis for Ceramides and Deoxyceramides

Principle

Ceramides are formed from dihydroceramides during the formation of sphingolipids. Ceramides are the building blocks for the synthesis of complex sphingolipids, and are hydrolyzed to sphingosine in the degradation pathway [26].

Procedure

1. Take 100 µl plasma into a 2 ml polypropylene tube
2. Add 1 ml Methanol / $CHCl_3$ (2+1) including 0.2 µl/ml internal standards and shake at 37°C for 1h

3. Add 0.5 ml $CHCl_3$
4. Add 200 μl alkaline water and centrifuge at full speed for 5 minutes
5. Remove upper phase with water pump and Pasteur pipette
6. Wash lower phase 3 times with 1 ml alkaline water; aspirate upper phase and protein interphase
7. Dry under N_2 for 15 – 20 minutes (samples can be stored at –20°C)

Mass Spectrometry for Sphingolipid and Deoxysphingolipid Analysis

Principle

Extracted lipids are solubilized and derivatized prior to separation on a C18 column (Uptispere, Interchim, France) and analysis by serial arrangement of a fluorescent detector (HP1046A, Hewlett Packard) and MS detector (LCMS-2010A, Shimadzu). Atmospheric pressure chemical ionization is used with non-natural C17 sphingosine (Avanti Polar Lipids) as an internal standard. MS data is analyzed using LC-MS solution (Shimadzu) and MS Processor version 11 (Advanced Chemistry Development) with normalization of quantified lipids for cell number and internal standards.

Procedure

1. Add 75 μl MetOH:EtOH:H_2O (85:50:15)
2. Add 5 μl freshly prepared OPA working solution (10 μl of 50 mg/ml o-Phtaldialdehyde (Sigma) in EtOH + 0.5 μl beta mercaptoethanol + 990 μl 3% Boric acid) to give 'derivation mix'
3. Allow to react for at least 1 h in the dark
4. Run on LC-MS
5. For the external standard sample use 10 μl of MS standard mix (C17SO, C18SO, C18SA, DoxSA, DeoxymethylSA and C20SA, 10 μl each in EtOH) + 140 μl MetOH:EtOH:H_2O (85:50:15) + 10 μl OPA working solution

Low-density lipoprotein (LDL) cholesterol (calculated)

Principle

LDL-C levels were calculated from the Friedewald formula. This formula is only accurate when serum triglycerides do not exceed 4.5 mmol/l.

Procedure

1. $\text{LDL-C} = \text{total cholesterol} - \text{HDL-C} - \frac{\text{Triglycerides}}{2.19}$

Non-HDL cholesterol (calculated)

Principle

Non-HDL-C levels estimate the cholesterol in all apolipoprotein B-containing particles including intermediate-density lipoprotein and very low-density lipoprotein in addition to LDL, and are increasingly recommended in clinical guidelines.

Procedure

1. Non-HDL-C = total cholesterol – HDL-C

Remnant cholesterol (calculated)

Principle

The cholesterol transported by remnant lipoproteins predicts the risk of coronary heart disease (CHD).

Procedure

1. Remnant Cholesterol = total cholesterol – HDL-C – LDL-C

Subclinical Atherosclerosis Assessments

40 patients with T1DM underwent Coronary Artery Calcification (CAC) Score determination by electron-beam CT using an automated program based on the Agatson method as a screening tool to identify subclinical atherosclerosis [27]. A CAC Score >10 was considered significant [28]. The carotid arteries were imaged in the remaining patients with T1DM with a Siemens Sequoia ultrasonography system (Siemens Medical Solutions, Mountain View, CA) with an 8- to 15-MHz linear array transducer. Examination included measurement of common and internal carotid artery flow velocities and Carotid Intima-Media thickness (CIMT) at each of 3 scan planes. Here a mean CIMT of 0.06 cm was considered significant [29].

Neuropathy Assessments

Participants were asked to complete a 38-point questionnaire (Neuropathy Symptom Profile) prior to clinical assessment. Patients with T1DM or T2DM were evaluated for the presence of diabetic sensory neuropathy using a modified Neuropathy Disability Score (NDS) with assessment of vibration perception using a 128-Hz tuning fork, ankle reflex testing, temperature differentiation and pinprick testing (Neurotips™, Owen Mumford Ltd., Oxford, United Kingdom). A score of 0 was given for a normal response and 1 for an abnormal response for each individual test component (except ankle reflex testing, where a score of 1 indicates presence with reinforcement, and 2 complete absence). Thus the maximum

score is 10, with an NDS of ≥ 3 indicative of peripheral neuropathy [30, 31]. All parameters were assessed by the same two examiners. The presence of large fibre neuropathy was evaluated by vibration perception threshold (VPT) testing using a Neurothesiometer (Horwell; Scientific Laboratory Supplies, Nottingham, UK) and nerve conduction studies for selected patients (Dantec Dynamics, Bristol, UK).

The presence of small fibre neuropathy was evaluated by quantitative sensory testing using a TSA-II NeuroSensory Analyzer (Medoc, Ramat-Yishai, Israel) and corneal confocal microscopy (CCM) using a Heidelberg Retinal Tomograph III Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany). The refraction of the objective lens was set at +12 dioptres and the camera adjusted for depth and resolution to facilitate optimal image acquisition. Six high-quality images at the level of the sub-basal nerve plexus were selected for image analysis for each patient. Automated image analysis was performed using Accemetrics32 (M.A. Dabbah, Imaging Science and Biomedical Engineering, The University of Manchester, UK). Neuropathy was diagnosed through CCM according to known age-adjusted normative values for corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD) and corneal nerve fibre length (CNFL) [32]. Assessments were performed by the same two examiners.

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Sciences software (SPSS 23.0, Illinois, USA). Results are expressed as mean ± SD unless stated otherwise where median and range are used because of skewed distribution. Statistical significance of the difference between 2 groups was performed by paired or 2-tailed independent t-tests. Normality of data was confirmed by Kolmogorov-Smirnov and Shapiro-Wilk tests. Homogeneity of variance was assessed using Levene's test. If more than 2 groups were considered, one-way analysis of variances (ANOVA) was used to test whether differences existed. Correlation between variables was assessed using Pearson's Coefficient following confirmation of linearity. Variables included in simple and multiple regression models were based on prior prediction of influencing factors. In all these tests, $P < 0.05$ was considered statistically significant. Where appropriate, Bonferroni correction was applied to the significance level.

1. High-density Lipoprotein Cholesterol Raising: Does it Matter?

Abstract

Purpose of review

Cardiovascular disease (CVD) is the leading cause of morbidity and premature mortality in Europe and the United States, and is increasingly common in developing countries. High-density lipoprotein cholesterol (HDL-C) is an independent risk factor for CVD and is superior to low-density lipoprotein cholesterol (LDL-C) as a predictor of cardiovascular events. The residual risk conferred by low HDL-C in patients with a satisfactory LDL-C was recently highlighted by the European Atherosclerosis Society. Despite the lack of randomized controlled trials, it has been suggested that raising the level of HDL-C should be considered as a therapeutic strategy in high-risk patients because of the strong epidemiological evidence, compelling biological plausibility, and both experimental and clinical research supporting its cardioprotective effects.

Recent findings

Three recent large randomized clinical trials investigating the effect of HDL-C raising with niacin and dalcetrapib in statin-treated patients failed to demonstrate an improvement in cardiovascular outcomes.

Summary

There is evidence to support the view that HDL functionality and the mechanism by which a therapeutic agent raises HDL-C are more important than plasma HDL-C levels. Future therapeutic agents will be required to improve this functionality rather than simply raising the cholesterol cargo.

Introduction

The prevention and treatment of atherosclerosis have historically been limited to lifestyle modification, smoking cessation, and reduction of blood pressure and low-density lipoprotein cholesterol (LDL-C) [17, 33, 34]. Although LDL-C lowering with statins has reduced cardiovascular events significantly, a substantial residual risk of 60–70% remains [35].

Most guidelines do not address high-density lipoprotein cholesterol (HDL-C) as a therapeutic target to reduce the residual cardiovascular risk in patients with satisfactory LDL-C levels [36]. The absence of unique HDL-C raising medications has made it difficult to define the benefit of raising HDL-C, but several clinical trials have suggested potential benefit [33, 37, 38].

Epidemiology

The inverse association between HDL-C concentration and cardiovascular disease (CVD) risk was first recognized in the 1970s [39, 40]. The Framingham Heart Study showed HDL-C to be an independent risk factor for CVD; an increase in HDL-C of 1 mg/dl (0.026 mmol/l) was associated with a risk reduction of 2–3% [1, 2]. The Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) and Systematic Coronary Risk Evaluation (SCORE) studies found HDL-C to be a better predictor of cardiovascular events than LDL-C [3, 41, 42].

The Emerging Risk Factors and Prospective Studies Collaborations showed that the inverse relationship between HDL-C and CVD risk persisted even when LDL-C was less than 2.6 mmol/l [3-6], suggesting significant residual cardiovascular risk after LDL-C reduction [7]. Furthermore, high HDL-C (>1.7 mmol/l) seems to be cardioprotective even when LDL-C is greater than 5.7 mmol/l [8].

In the US Physicians' Health Study, low total cholesterol did not protect against myocardial infarction (MI) in the presence of low HDL-C [3, 43]. Moreover, up to 55% of patients hospitalized with CVD have HDL-C less than 1.0 mmol/l on admission [33, 44].

The Monica Risk, Genetics, Archiving and Monograph (MORGAM) Project showed an association between higher HDL-C levels and up to a 20% risk reduction for stroke [45]. This inverse relationship suggests that, in addition to inhibiting chronic inflammation, HDL may act to reduce the acute inflammation implicated in cerebrovascular events.

Although Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) found that HDL-C did not predict first nonfatal MI and stroke in patients already on high-dose statin treatment [46], a recent meta-analysis reported that statin monotherapy did not alter the correlation between HDL-C level and cardiovascular risk [47]. HDL-C in patients receiving statins also predicted major cardiovascular events in the Treating to New Targets (TNT) study cohort [3, 48]. This is important because some studies have reported that nearly 80% of statin-treated patients have low HDL-C levels [49].

Low HDL-C appears to be of greater importance in patients at high cardiometabolic risk and is amongst the factors that currently favour a decision to further reduce LDL-C levels [50, 51]. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study showed an increased number of cardiovascular events in diabetic patients with persistently low HDL-C and high triglyceride levels, despite a mean LDL-C less than 2.1 mmol/l [52, 53].

The secondary prevention Veteran Affairs High-Density Lipoprotein Intervention Trial (VA-HIT) demonstrated that events could be reduced in patients with low HDL-C – the beneficial effect of gemfibrozil was related to increases in HDL-C but not changes in triglycerides or LDL-C [54]. The Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol 6 – HDL and LDL Treatment Strategies in Atherosclerosis (ARBITER 6-HALTS) study also suggested that raising HDL-C should be the next therapeutic target [55, 56].

HDL Heterogeneity

Although HDL-C levels are a strong biomarker for assessing CVD risk, they do not predict either HDL functionality or composition [10]. HDL metabolism and therapeutic targets are summarized in Figure 1.1 [12, 14, 57].

Plasma HDL is a heterogeneous collection of small discoid and spherical particles that are functionally diverse and differ in composition, size and electrophoretic mobility [58]. HDL particles can be separated by sequential ultracentrifugation into large, light, lipid-rich HDL2 and small, dense, protein-rich HDL3 [59]. HDL2 and HDL3 can be further subfractionated by non-denaturing polyacrylamide gradient gel electrophoresis into five distinct subpopulations of decreasing size: HDL2b, HDL2a, HDL3a, HDL3b and HDL3c [58].

These HDL subfractions may have different functional properties, but attempts to clarify their effects on cardiovascular risk have led to inconclusive results [3]. Population studies suggest that HDL2 is more cardioprotective than HDL3, but HDL3 has been reported to be superior in its capacity to inhibit vascular cell adhesion molecule 1 (VCAM-1) expression in endothelial cells [60]. The distinct anti-inflammatory properties of HDL2 and HDL3 may be

related to differences in their protein and phospholipid concentrations rather than their size [61].

Identification of subclasses of HDL displaying specific biological functions may lead to their use as biomarkers for cardiovascular risk or in the assessment of novel HDL-targeted therapies [62]. Promising biomarkers of HDL functionality and cardiovascular risk include plasma myeloperoxidase (MPO), paraoxonase-1 (PON1) [63, 64], apolipoprotein AI (apo AI), lecithin-cholesterol acyltransferase (LCAT), lipoprotein phospholipase A2, serum amyloid A (SAA) and apolipoprotein J [12, 65].

HDL Functions

Discoid lipid-poor HDL particles promote cholesterol efflux from subendothelial macrophages and foam cells [66], via interactions with ATP-binding cassette transporter A1 (ABCA1), after esterification by LCAT. The enzyme PON1 enhances cholesterol efflux [67]. Subsequently, HDL particles deliver their cholesterol to the liver directly, via scavenger receptor-B1 (SR-B1), or indirectly, via transfer to very low-density lipoprotein (VLDL) or LDL particles by the action of cholesteryl ester transfer protein (CETP) [63]. These apo B-containing lipoproteins are taken up by the liver via the LDL receptor [1]. Facilitating the efflux of excess cholesterol from macrophages in the arterial wall and its return to the liver for excretion in the bile and faeces is the best known of HDL's cardioprotective functions [62]. This process of cholesterol clearance is known as reverse cholesterol transport (RCT) [68].

Recently, a variety of other HDL functions have been described (Figure 1.2) [12, 69-71].

The increased expectation that modification of HDL might reduce CVD risk through these pleiotropic effects seems confounded by the recent reports from genetic and pharmacological studies suggesting that higher levels of plasma HDL-C are not associated with decreased CVD risk [9].

HDL Dysfunction

HDL dysfunction is suggested by the observation that high HDL-C levels do not always protect against CVD [11]. HDL can undergo modification in structure and composition to become dysfunctional in conditions associated with systemic inflammation and oxidative stress [72, 73]. Dysfunctional HDL is proinflammatory and contains oxidized phospholipids and lysophospholipids, as well as proinflammatory proteins, such as SAA and ceruloplasmin. The genesis of dysfunctional HDL has been attributed to oxidation, chlorination or nitration of apo AI through non-enzymatic glycation, homocysteinylation, and reactions with metal

ions, peroxyl and hydroxyl radicals, aldehydes, MPO-generated oxidants, elastase, lipoxygenase, and phosphorylase A2 [62]. Dysfunctional HDL may promote the transfer of lipid hydroperoxides to apo B-containing lipoproteins and actually promote the VLDL and LDL oxidation required for atherosclerotic lesion formation [11]. Proinflammatory HDL-C has been associated with increased carotid intima-media thickness and an increased risk for adverse clinical outcomes [74]. HDL from patients with coronary artery disease does not prevent LDL oxidation [75]. On the other hand, raising the level of functional HDL particles by increasing their hepatic production or by HDL infusion results in atheroprotective effects [57].

HDL function can be measured *in vitro* [13]. The capacity of HDL-C to promote cholesterol efflux from macrophages is a recognized metric of HDL function and has recently been shown to have a strong inverse relationship with both carotid intima-media thickness and the presence of angiographically confirmed coronary artery disease, independent of HDL-C levels [76, 77]. Determining HDL function may identify patients with normal or low HDL-C at high risk of CVD. Future therapeutic agents targeting HDL may be required to improve its functionality rather than simply raising its cholesterol cargo [12].

Genetics

Common genetic variants associated with HDL-C, but not other lipoprotein traits, were not associated with MI in a recent Mendelian randomization study [9], suggesting that raising HDL-C pharmacologically will not necessarily translate into a reduced cardiovascular risk.

The antiatherogenic action of apo AI is well established [78]. Mutations of *Apo AI* could explain the functional heterogeneity of HDL particles [3]. Several genetic syndromes with reduced HDL-C and apo AI are not associated with an increased risk of premature CVD [62]. Heterozygosity for the Milano or Paris mutations of the *Apo AI* gene, despite HDL-C less than 0.5 mmol/l, does not confer early CVD risk [79]. Deficiency of plasma LCAT does not increase CVD risk despite HDL-C less than 0.4 mmol/l [80]. Tangier disease, caused by mutations in the ABCA1 transporter gene, in which HDL and apo AI concentrations are virtually undetectable, is not associated with the increase in CVD expected from such an extreme phenotype [63]. Genetic deficiency of CETP increases HDL-C but would also be expected to impair RCT [81]. The mechanism by which HDL-C is increased is critical in determining whether it reduces atherosclerosis. Thus, increases in HDL-C through downregulation of SR-B1 by oestrogens and CETP activity modulation by CETP inhibitors may not reduce cardiovascular risk.

Lifestyle Modification

Lifestyle interventions have been shown to be effective in increasing HDL-C levels and decreasing cardiovascular risk [3]. Most current guidelines for the management of dyslipidaemia in patients with low HDL-C levels recommend a change in lifestyle, focussing on smoking cessation, regular exercise, weight loss, dietary modifications and modest alcohol consumption [82]. In patients already on statin therapy, these dietary and lifestyle changes can raise HDL-C levels by an additional 5–10% [83]. A Mediterranean-style diet rich in fruits and vegetables and high in polyunsaturated fats improves the anti-inflammatory properties of HDL, decreases LDL-C, increases HDL-C and reduces cardiovascular events [84]. A low glycaemic index carbohydrate diet has been shown to improve HDL-C, glycaemic control and inflammatory markers [85]. Weight loss achieved through exercise may be more effective at raising HDL-C concentrations than dieting [86], but it is again difficult to attribute the reduction in cardiovascular risk to increases in HDL-C alone [87]. Smoking cessation increases HDL-C by 3–5.6% without changes in LDL-C, total cholesterol or triglyceride levels. It also favours RCT, decreases CETP and can reduce oxidative stress by improving endothelial function [88]. Moderate alcohol consumption can increase HDL-C by as much as 12% [4]. It remains unclear how much this contributes to the reduced incidence of CVD with moderate consumption of alcohol [89, 90]. Alcohol may interfere with CETP activity, reducing the transfer of cholesteryl ester from HDL [4].

Current Therapies

Lipid-lowering treatment with hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) can achieve a relative risk reduction for CVD of 25–40% [82, 91], which is accepted to be secondary to LDL-C reduction, but may also be related to increases in HDL-C or HDL functionality. Statins can increase HDL-C by 5–15% [92], with a comparable increase in apo AI levels. A Study To Evaluate the Effect of Rosuvastatin on Regression of Coronary Atherosclerosis (ASTEROID) suggested that statin-induced reductions in atheroma volume are because of increases in HDL-C in addition to reductions in LDL-C. In the Lipoprotein and Coronary Atherosclerosis Study (LCAS), fluvastatin reduced angiographic progression in patients with low HDL-C levels significantly compared with those with high HDL-C levels [93]. Statins increase HDL-C by activating peroxisome proliferator-activated receptor-α (PPAR-α), increasing the expression of apo AI and apo AII [94]. They may also increase HDL-C by reducing hepatic CETP expression and, thus, CETP-dependent transfer of cholesterol from HDL to VLDL. As statins lower MPO levels, they may also preserve HDL function by creating a less inflamed environment [95]. There is also some evidence that PON1 activity may be augmented by atorvastatin [96].

If concomitant hypertriglyceridaemia is present, most guidelines recommend consideration of a fibrate [33]. The hallmarks of fibrate therapy are a 30–50% reduction in plasma triglyceride levels and a 10–15% increase in HDL-C levels [97, 98]. Despite this, the

beneficial effect of fibrates on all-cause and cardiac mortality remains controversial [98]. Fibrates act as PPAR-α agonists. They also increase HDL-C by reducing the CETP-dependent transfer of cholesterol from HDL to apo B-containing lipoprotein particles. Their effect on lipid modification, however, does not appear to be consistent [54, 99].

Bile acid sequestrants and cholesterol uptake inhibitors such as ezetimibe reduce cholesterol absorption. They have minimal effects on HDL-C. Omega-3 fatty acids reduce triglycerides in a dose-dependent manner, but their effect on cardiovascular events appears to be independent of HDL [100].

Niacin (Nicotinic Acid)

The interest in raising HDL-C has brought renewed focus on the oldest and most effective agent in increasing HDL-C. Niacin was first integrated in clinical practice in the 1960s, when it was the first lipid-modifying agent shown to have an effect on cardiovascular endpoints [7]. Despite promising results, high rates of intolerance and adverse effects have precluded its widespread use.

Niacin increases HDL-C by 23%, and reduces LDL-C by 20% and triglyceride levels by 40% [51]. In a recent meta-analysis of 11 randomized controlled trials, a therapeutic strategy including niacin was shown to reduce major coronary events by 25%, stroke by 26%, and all cardiovascular events by 27%. The cardiovascular benefit of niacin appeared to be greater in patients with diabetes and metabolic syndrome despite mild adverse effects on glucose levels [101].

Although a number of trials (Table 1.1) have demonstrated benefit from adding niacin to other lipid-lowering drugs [38, 56, 102-105], it is difficult to attribute the benefit of niacin to its HDL-C-raising properties as opposed to its ability to lower LDL-C. Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides and Impact on Global Health Outcomes (AIM-HIGH) showed no benefit from adding niacin to simvastatin [33] although it is considered to be underpowered and have other design flaws [106]. Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE), the largest study of niacin ever undertaken, showed no benefit of adding niacin and laropiprant (Tredaptive) to simvastatin, but did demonstrate significant side-effects [107]. Although HPS2-THRIVE had no cutoff for HDL-C and LDL-C was low at less than 2 mmol/l before randomization, no type of patient has yet been identified who benefitted from treatment [108].

Future Agents

Interest in the inhibition of CETP was stimulated by a 1985 publication describing a Japanese population in which a low-activity genetic variant of CETP was associated with elevated HDL-C and relatively low rates of CVD [33].

The first oral CETP inhibitor to be assessed in major clinical trials was torcetrapib. Despite promising results in phase I and II studies, increases in HDL-C of 72% and decreases in LDL-C of 25% and triglycerides of 9%, the large-scale phase III Investigation of Lipid Level Management to Understand its Impact in Atherosclerosis Events (ILLUMINATE) trial was terminated early because the torcetrapib and atorvastatin group experienced increased all-cause mortality and cardiovascular events compared with recipients of atorvastatin alone [109]. This has been attributed to CETP-independent angiotensin–aldosterone activation with adverse effects on blood pressure. However, CVD rates were lower in torcetrapib-treated patients whose HDL-C increased above the median [110]. HDL isolated from torcetrapib-treated patients showed normal or enhanced promotion of cholesterol efflux [111].

The second CETP inhibitor to progress to phase III studies was dalcetrapib, which associates differently with CETP and has no angiotensin–aldosterone-stimulating or hypertensive effects. Disappointingly, the dal-OUTCOMES (efficacy and safety of dalcetrapib in patients with recent acute coronary syndrome) trial showed no effect from dalcetrapib on cardiovascular events, despite raising HDL-C by 25% [112]. Dalcetrapib was discontinued from clinical development in 2012 following the second prespecified interim analysis of primary end-point events (death from coronary heart disease, nonfatal myocardial infarction, ischaemic stroke, unstable angina or cardiac arrest with resuscitation).

A third agent, anacetrapib, has greater effects on HDL-C and LDL-C. Like dalcetrapib, it does not appear to cause hypertension or have any other 'off-target' effects. At present, there is no evidence to suggest any harm from complete CETP inhibition with anacetrapib (or its modulation with dalcetrapib) [113, 114]. The phase III study Determining the Efficacy and tolerability of CETP Inhibition with Anacetrapib (DEFINE) examined its effect in high-risk patients already on statin therapy [113]. Anacetrapib reduced LDL-C by 40% and increased HDL-C by 138%. Cardiovascular outcomes are currently being investigated [100]. Although the development of dalcetrapib has been discontinued, a fourth CETP inhibitor, evacetrapib, is in development [115].

Drugs in development are shown in Table 1.2 [100, 112, 115-122].

Conclusion

Lowering LDL-C has been the primary focus in lipid modification for the prevention and treatment of atherosclerosis [17]. The ARBITER 6-HALTS study and others implied that raising HDL-C should be the next target to ameliorate the progression of CVD [55], but increases in HDL-C may not result in the cardiovascular benefit suggested by prospective observational studies [123]. To date, HDL-based therapy to reduce the residual risk of CVD remains a largely unfulfilled promise [124]. Although raising the level of functional HDL particles either by increasing their hepatic production or by HDL infusion appears promising [57], there is limited evidence that any of the clinical endpoints measured to date (plaque volume and inflammatory state of macrophages) are correlated with decreased events [125].

There is increasing evidence that HDL-C levels are not necessarily directly correlated to HDL particle function [126]. As we understand the structural components of HDL and exact roles of each component in HDL function, traditional lipid panel studies will likely be superseded by assays of HDL function or measurement of biomarkers associated with dysfunctional HDL [62]. There remains cautious optimism that CETP inhibition may be a viable option to reduce cardiovascular risk, but the concern that the inhibition of CETP might lead HDL to become dysfunctional has not yet been fully allayed.

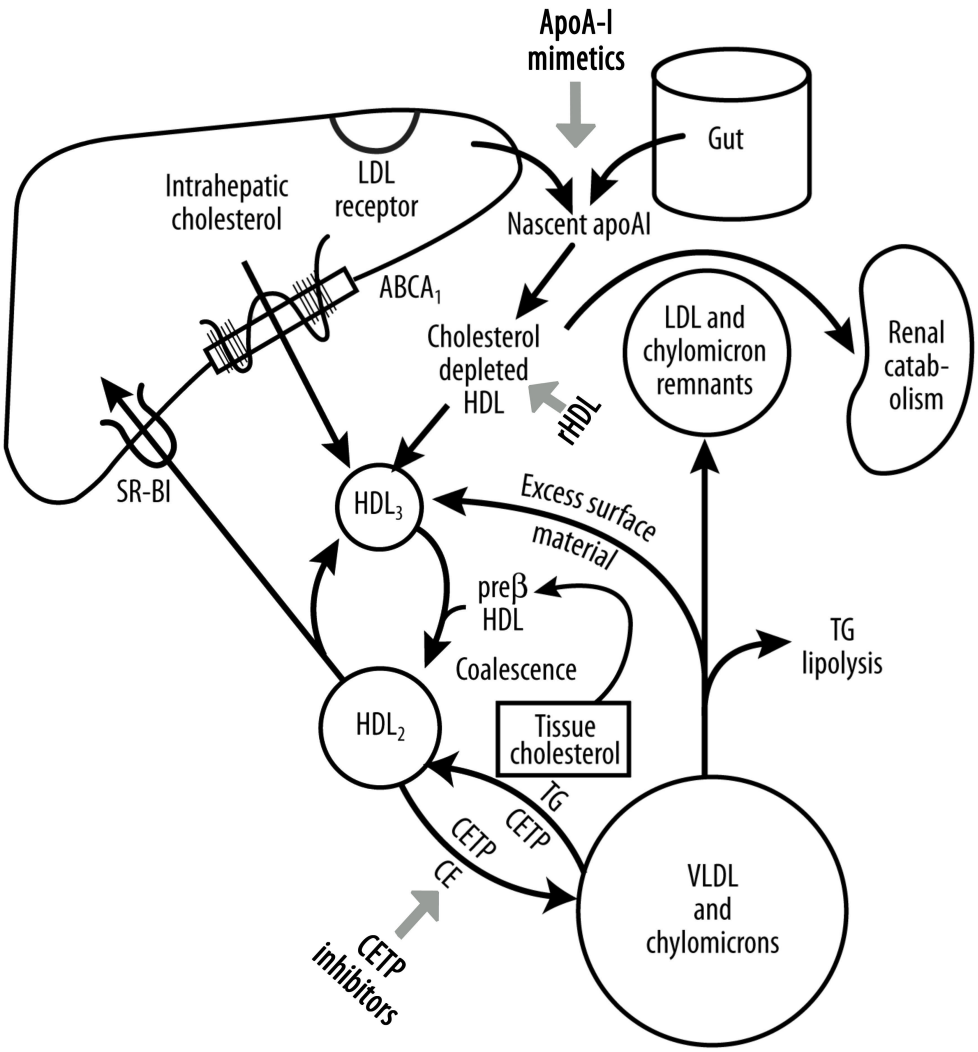

Figure 1.1: HDL assembly begins with the secretion of apolipoprotein AI (apo AI) in the liver and intestine. Nascent discoid lipid-poor HDL particles are formed by the extracellular acquisition of other apolipoproteins, lipids and phospholipids. HDL is continually remodelled by plasma and cell surface enzymes in processes mediated by ATP-binding cassette subfamily G member 1 (ABCG1), hepatic lipase, endothelial lipase, cholesteryl ester transfer protein (CETP), and phospholipid transfer protein. Interaction between HDL and the cell membrane mediated by scavenger receptor-B1 (SR-B1) promotes the hydrolysis of intracellular cholesteryl ester (CE) to form free cholesterol and its passage to the cell membrane, which it crosses to be incorporated into preβ HDL and smaller HDL3 particles directly or via SR-B1 and ATP-binding cassette transporter A1 (ABCA1). The removal of CE from HDL is attenuated by CETP and hepatic SR-BI. *rHDL, reconstituted HDL.*

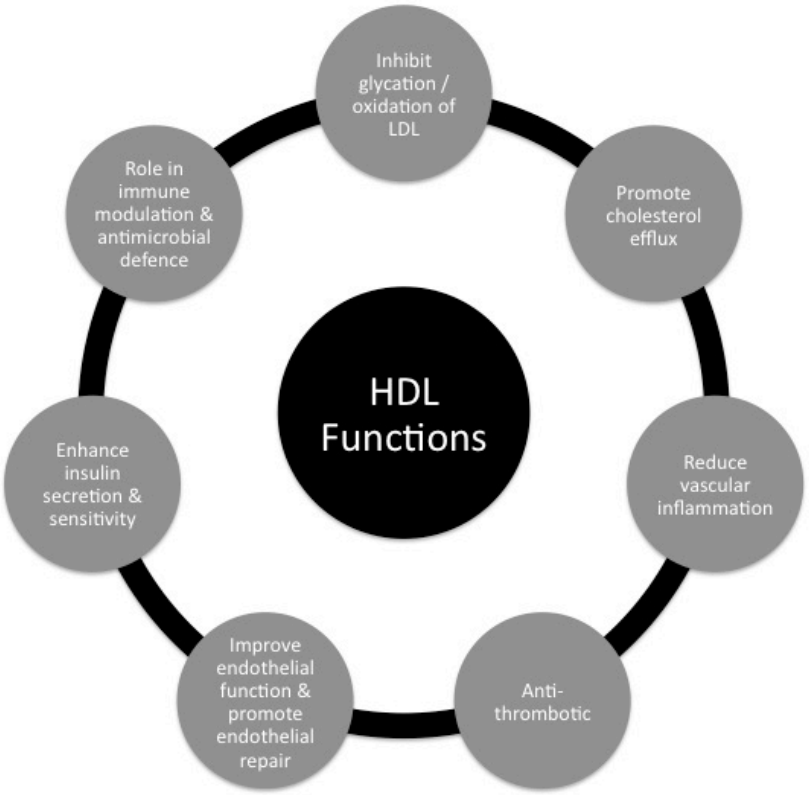

Figure 1.2: Circulating LDL must be modified by glycation or oxidation before its uptake by macrophages in the arterial wall is rapid enough to excite foam cell formation and inflammation. HDL can inhibit this atherogenic modification *in vitro*. Both spherical HDL from human plasma and discoidal rHDL have been shown to inhibit vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression. These anti-inflammatory effects appear to be mediated primarily by apo AI, with a smaller contribution from phospholipids and through activation of ABCA1-dependent signalling pathways. Other anti-inflammatory effects include inhibition of the reactive oxygen species/nuclear factor kappa B signalling pathway and inhibition of monocyte activation and adhesion to the endothelium by downregulating the expression of monocyte chemoattractant protein-1. Some of HDL's anti-inflammatory effects are thought to be because of PON1 and phospholipase A2 associated with it. HDL contributes to the maintenance of vascular endothelial function through the SR-BI- dependent induction of endothelial nitric oxide synthase and enhanced prostacyclin synthesis. HDL also inhibits endothelial cell apoptosis and promotes re-endothelialization after injury by preserving the viability of phagocytic macrophages and promoting cholesterol efflux. The antithrombotic activities of HDL include the inhibition of platelet activation and aggregation, activation of protein C and S, reduced von Willebrand factor levels, and actions on tissue factor and factors X, Va and VIIIa. More recently, HDL has been shown to have a potential role in diabetes pathophysiology and its complications. *ABCA1, ATP-binding cassette transporter A1; rHDL, reconstituted HDL.*

Trial	Year	Participants	Treatment Arms	Study Design	Outcome
Coronary Drug Project (CDP)	1975 1986	8,341 men with previous myocardial infarction	Oestrogen *vs.* dextrothyroxine *vs.* clofibrate *vs.* niacin *vs.* placebo	Randomized, placebo-controlled trial; end-points recurrent nonfatal MI at 6 years & total mortality at 15 years	Niacin significantly decreased nonfatal recurrent myocardial infarction at 6 years & total mortality at 15 years
Cholesterol Lowering Atherosclerosis Study (CLAS)	1987	162 non-smoking men aged 40-59 years with previous coronary bypass surgery	Colestipol + niacin *vs.* Placebo	2 year randomized, placebo-controlled, angiographic trial	43% reduction in LDL-C; 37% elevation of HDL-C; Atherosclerosis regression in 16.2% of colestipol-niacin treated *vs.* 2.4% placebo treated
Familial Atherosclerosis Treatment Study (FATS)	1990	146 men with apolipoprotein B levels >125 mg/dl, documented coronary artery disease, & family history of vascular disease	Lovastatin + colestipol *vs.* niacin + colestipol *vs.* placebo	2 ½ year randomized, double-blind study; arteriography at baseline & after treatment	LDL-C decreased by 25% & HDl-C increased by 38%; coronary lesion progression seen in 39%
HDL-Atherosclerosis Treatment Study (HATS)	2001	160 patients with coronary disease, low HDL-C and normal LDL-C	Simvastatin + niacin *vs.* vitamins *vs.* simvastatin-niacin + antioxidants *vs.* placebo	3 year double-blind trial; end points arteriographic evidence of change in coronary stenosis & first cardiovascular event	Frequency of clinical end point 24% with placebo; 3% with simvastatin-niacin; 21% in antioxidant group; 14% in simvastatin-niacin + antioxidants
Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER)	2004 2006	167 patients with known CHD and HDL-C <1.16mmol/l on statin (ARBITER 2) 130 subjects completing ARBITER 2 (ARBITER 3)	Extended release niacin *vs.* placebo	Randomized placebo-controlled trials; primary end-point change in carotid intima-media thickness at 12 (ARBITER 2) & 24 months (ARBITER 3)	HDL-C increased from 1.01-1.22mmol/l; progression of atherosclerosis slowed by 68% (ARBITER 2); HDL-C increased from 1.02-1.26 mmol/l; additional regression of carotid intima-media thickness of 0.041 mm
Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol 6 – HDL and LDL Treatment Strategies in Atherosclerosis (ARBITER 6-HALTS)	2010	315 patients with coronary heart disease or equivalent with LDL-C <100mg/dl & HDL-C <55mg/dl while receiving stable statin treatment	Ezetimibe *vs.* extended-release niacin	14 month randomized study; primary end point change in carotid intima-media thickness	Terminated early on basis of pre-specified interim analysis showing superiority of niacin over ezetimibe on change in carotid intima-media thickness

Table 1.1: Trials investigating the effect of adding niacin to other lipid-lowering agents. *CHD, coronary heart disease; HDL-C, high-density lipoprotein cholesterol; LDL-C low-density lipoprotein cholesterol; MI: myocardial infarction*

Class	Mechanism of Action	Examples	Developer	Status
CETP Inhibitors	Inhibit CETP to increase circulating HDL-C and lower LDL-C	Torcetrapib	Pfizer	Discontinued 2006
		Dalcetrapib	Hoffmann-La Roche	Discontinued 2012
		Evacetrapib	Lilly	Phase III trial ACCELERATE to present in 2015
		Anacetrapib	Merck	Phase III trial REVEAL to present in 2017
ApoA-I mimetics	Stimulate cholesterol efflux from macrophages via ABCAI and associate with HDL to induce pre-β HDL formation	D-4F	Novartis	Phase II trials
Stimulator of ApoA-I gene transcription	Increases ApoA-I, pre-β HDL, & HDL functionality	RVX-208	Resverlogix	Phase I trials
Liver Receptor X (LXR) Agonists	Activate RCT	DMHCA	Wyeth	Phase I trials
rHDL	Infusion of reconstituted or recombinant HDL particles	ApoA-I Milano-phospholipid complex	The Medicines Company	Phase II trials
		Wild-type ApoA-I-phospholipid complex	Cerenis Therapeutics	Phase II trials

Table 1.2: HDL-modifying drugs in development. *ABCA1, ATP-binding membrane cassette transporter A1; ACCELERATE, Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition With Evacetrapib in Patients at a High-Risk for Vascular Outcomes; apo A1, apolipoprotein A1; CETP, cholesteryl ester transfer protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RCT, reverse cholesterol transport; REVEAL, Randomized Evaluation of the Effects of Anacetrapib Through Lipid-modification.*

High-density lipoprotein cholesterol raising: does it matter?

Schofield JD, France M, Ammori B, Liu Y, Soran H.

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High-density lipoprotein cholesterol raising: does it matter?

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Purpose of review
Cardiovascular disease (CVD) is the leading cause of morbidity and premature mortality in Europe and the United States, and is increasingly common in developing countries. High-density lipoprotein cholesterol (HDL-C) is an independent risk factor for CVD and is superior to low-density lipoprotein cholesterol (LDL-C) as a predictor of cardiovascular events. The residual risk conferred by low HDL-C in patients with a satisfactory LDL-C was recently highlighted by the European Atherosclerosis Society. Despite the lack of randomized controlled trials, it has been suggested that raising the level of HDL-C should be considered as a therapeutic strategy in high-risk patients because of the strong epidemiological evidence, compelling biological plausibility, and both experimental and clinical research supporting its cardioprotective effects.

Recent findings
Three recent large randomized clinical trials investigating the effect of HDL-C raising with niacin and dalcetrapib in statin-treated patients failed to demonstrate an improvement in cardiovascular outcomes.

Summary
There is evidence to support the view that HDL functionality and the mechanism by which a therapeutic agent raises HDL-C are more important than plasma HDL-C levels. Future therapeutic agents will be required to improve this functionality rather than simply raising the cholesterol cargo.

Keywords
cardiovascular disease, HDL functionality, HDL-cholesterol

INTRODUCTION

The prevention and treatment of atherosclerosis have historically been limited to lifestyle modification, smoking cessation, and reduction of blood pressure and low-density lipoprotein cholesterol (LDL-C) [1–3]. Although LDL-C lowering with statins has reduced cardiovascular events significantly, a substantial residual risk of 60–70% remains [4].

Most guidelines do not address high-density lipoprotein cholesterol (HDL-C) as a therapeutic target to reduce the residual cardiovascular risk in patients with satisfactory LDL-C levels [5]. The absence of unique HDL-C raising medications has made it difficult to define the benefit of raising HDL-C, but several clinical trials have suggested potential benefit [1,6,7].

EPIDEMIOLOGY

The inverse association between HDL-C concentration and cardiovascular disease (CVD) risk was first recognized in the 1970s [8,9]. The Framingham Heart Study showed HDL-C to be an independent risk factor for CVD; an increase in HDL-C of 1 mg/dl (0.026 mmol/l) was associated with a risk reduction of 2–3% [10,11]. The Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) and Systematic Coronary Risk Evaluation (SCORE) studies found HDL-C to be a better predictor of cardiovascular events than LDL-C [12–14].

The Emerging Risk Factors and Prospective Studies Collaborations showed that the inverse relationship between HDL-C and CVD risk persisted even when LDL-C was less than 2.6 mmol/l [13,15–17], suggesting significant residual cardiovascular risk after LDL-C reduction [18]. Furthermore, high

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KEY POINTS

- HDL-C is an independent risk factor for CVD.
- HDL has a variety of cardioprotective functions in addition to its role in reverse cholesterol transport.
- Recent trials investigating the effect of HDL-C raising with niacin and dalcetrapib in statin-treated patients have failed to demonstrate an improvement in cardiovascular outcomes.
- HDL-C is not a reliable biomarker of HDL function.
- Future therapeutic agents targeting HDL may be required to improve its functionality rather than simply raising concentrations.

HDL-C (>1.7 mmol/l) seems to be cardioprotective even when LDL-C is greater than 5.7 mmol/l [19].

In the US Physicians' Health Study, low total cholesterol did not protect against myocardial infarction (MI) in the presence of low HDL-C [13,20]. Moreover, up to 55% of patients hospitalized with CVD have HDL-C less than 1.0 mmol/l on admission [1,21].

The Monica Risk, Genetics, Archiving and Monograph (MORGAM) Project showed an association between higher HDL-C levels and up to a 20% risk reduction for stroke [22]. This inverse relationship suggests that, in addition to inhibiting chronic inflammation, HDL may act to reduce the acute inflammation implicated in cerebrovascular events.

Although Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) found that HDL-C did not predict first nonfatal MI and stroke in patients already on high-dose statin treatment [23], a recent meta-analysis reported that statin monotherapy did not alter the correlation between HDL-C level and cardiovascular risk [24]. HDL-C in patients receiving statins also predicted major cardiovascular events in the Treating to New Targets (TNT) study cohort [13,25]. This is important because some studies have reported that nearly 80% of statin-treated patients have low HDL-C levels [26].

Low HDL-C appears to be of greater importance in patients at high cardiometabolic risk and is amongst the factors that currently favour a decision to further reduce LDL-C levels [27,28]. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study showed an increased number of cardiovascular events in diabetic patients with persistently low HDL-C and high triglyceride levels, despite a mean LDL-C less than 2.1 mmol/l [29,30].

The secondary prevention Veteran Affairs High-Density Lipoprotein Intervention Trial (VA-HIT) demonstrated that events could be reduced in patients with low HDL-C – the beneficial effect of gemfibrozil was related to increases in HDL-C but not changes in triglycerides or LDL-C [31]. The Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol 6 – HDL and LDL Treatment Strategies in Atherosclerosis (ARBITER 6-HALTS) study also suggested that raising HDL-C should be the next therapeutic target [32,33].

HDL HETEROGENEITY

Although HDL-C levels are a strong biomarker for assessing CVD risk, they do not predict either HDL functionality or composition [34]. HDL metabolism and therapeutic targets are summarized in Fig. 1 [35,36▪▪,37].

Plasma HDL is a heterogeneous collection of small discoid and spherical particles that are functionally diverse and differ in composition, size and electrophoretic mobility [38]. HDL particles can be separated by sequential ultracentrifugation into large, light, lipid-rich HDL2 and small, dense, protein-rich HDL3 [39]. HDL2 and HDL3 can be further subfractionated by nondenaturing polyacrylamide gradient gel electrophoresis into five distinct subpopulations of decreasing size: HDL2b, HDL2a, HDL3a, HDL3b and HDL3c [38].

These HDL subfractions may have different functional properties, but attempts to clarify their effects on cardiovascular risk have led to inconclusive results [13]. Population studies suggest that HDL2 is more cardioprotective than HDL3, but HDL3 has been reported to be superior in its capacity to inhibit vascular cell adhesion molecule 1 (VCAM-1) expression in endothelial cells [40]. The distinct anti-inflammatory properties of HDL2 and HDL3 may be related to differences in their protein and phospholipid concentrations rather than their size [41].

Identification of subclasses of HDL displaying specific biological functions may lead to their use as biomarkers for cardiovascular risk or in the assessment of novel HDL-targeted therapies [42]. Promising biomarkers of HDL functionality and cardiovascular risk include plasma myeloperoxidase (MPO), paraoxonase-1 (PON1) [43,44], apolipoprotein A-I (apoA-I), lecithin-cholesterol acyltransferase (LCAT), lipoprotein phospholipase A2, serum amyloid A (SAA) and apolipoprotein J [36▪▪,45].

HDL FUNCTIONS

Discoid lipid-poor HDL particles promote cholesterol efflux from subendothelial macrophages and

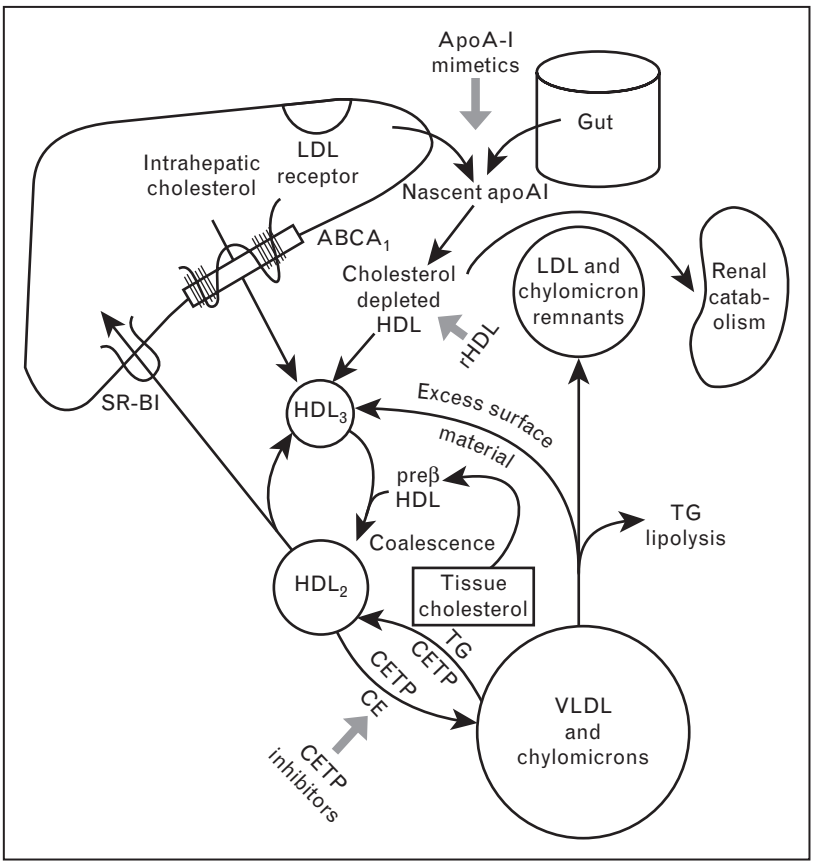

FIGURE 1. HDL assembly begins with the secretion of apolipoprotein A-I (apoA-I) in the liver and intestine. Nascent discoid lipid-poor HDL particles are formed by the extracellular acquisition of other apolipoproteins, lipid and phospholipids. HDL is continually remodelled by plasma and cell surface enzymes in processes mediated by ATP-binding cassette subfamily G member 1 (ABCG1), hepatic lipase, endothelial lipase, cholesteryl ester transfer protein (CETP), and phospholipid transfer protein. Interaction between HDL and the cell membrane mediated by scavenger receptor-B1 (SR-B1) promotes the hydrolysis of intracellular cholesteryl ester (CE) to form free cholesterol and its passage to the cell membrane, which it crosses to be incorporated into preβ HDL and smaller HDL3 particles directly or via SR-B1 and ATP-binding cassette transporter A1 (ABCA1). The removal of CE from HDL is attenuated by CETP and hepatic SR-BI. rHDL, reconstituted HDL. Adapted with permission [35].

foam cells [46], via interactions with ATP-binding cassette transporter A1 (ABCA1), after esterification by LCAT. The enzyme PON1 enhances cholesterol efflux [47]. Subsequently, HDL particles deliver their cholesterol to the liver directly, via scavenger receptor-B1 (SR-B1), or indirectly, via transfer to very low-density lipoprotein (VLDL) or LDL particles by the action of cholesteryl ester transfer protein (CETP) [43]. These apoB-containing lipoproteins are taken up by the liver via the LDL receptor [10]. Facilitating the efflux of excess cholesterol from macrophages in the arterial wall and its return to the liver for excretion in the bile and faeces is the best known of HDL's cardioprotective functions [42]. This process of cholesterol clearance is known as reverse cholesterol transport (RCT) [48].

Recently, a variety of other HDL functions have been described (Fig. 2) [36▪▪,49–58,59▪].

The increased expectation that modification of HDL might reduce CVD risk through these pleiotropic effects seems confounded by the recent reports from genetic and pharmacological studies suggesting that higher levels of plasma HDL-C are not associated with decreased CVD risk [60▪▪].

HDL DYSFUNCTION

HDL dysfunction is suggested by the observation that high HDL-C levels do not always protect against CVD [61]. HDL can undergo modification in structure and composition to become dysfunctional in conditions associated with systemic inflammation and oxidative stress [62,63]. Dysfunctional HDL is proinflammatory and contains oxidized phospholipids and lysophospholipids, as well as proinflammatory proteins, such as SAA and ceruloplasmin. The genesis of dysfunctional HDL has been attributed

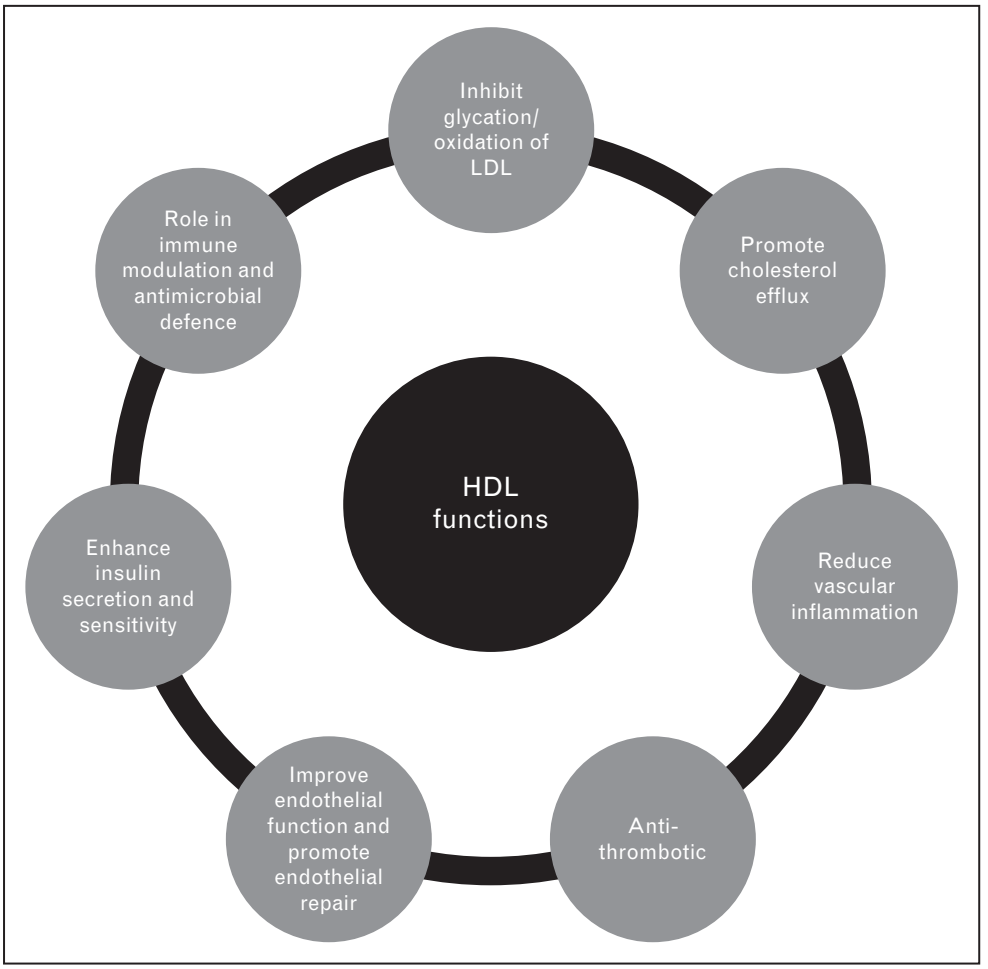

FIGURE 2. Circulating LDL must be modified by glycation or oxidation before its uptake by macrophages in the arterial wall is rapid enough to excite foam cell formation and inflammation. HDL can inhibit this atherogenic modification *in vitro*. Both spherical HDL from human plasma and discoidal rHDL have been shown to inhibit vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression. These anti-inflammatory effects appear to be mediated primarily by apoA-I, with a smaller contribution from phospholipids and through activation of ABCA1-dependent signalling pathways. Other anti-inflammatory effects include inhibition of the reactive oxygen species/nuclear factor kappa B signalling pathway and inhibition of monocyte activation and adhesion to the endothelium by downregulating the expression of monocyte chemoattractant protein-1. Some of HDL's anti-inflammatory effects are thought to be because of PON1 and phospholipase A2 associated with it. HDL contributes to the maintenance of vascular endothelial function through the SR-BI-dependent induction of endothelial nitric oxide synthase and enhanced prostacyclin synthesis. HDL also inhibits endothelial cell apoptosis and promotes re-endothelialization after injury by preserving the viability of phagocytic macrophages and promoting cholesterol efflux. The antithrombotic activities of HDL include the inhibition of platelet activation and aggregation, activation of protein C and S, reduced von Willebrand factor levels, and actions on tissue factor and factors X, Va and VIIIa. More recently, HDL has been shown to have a potential role in diabetes pathophysiology and its complications. ABCA1, ATP-binding cassette transporter A1; rHDL, reconstituted HDL.

to oxidation, chlorination or nitration of apoA-I through nonenzymatic glycation, homocysteinylation, and reactions with metal ions, peroxyl and hydroxyl radicals, aldehydes, MPO-generated oxidants, elastase, lipoxygenase, and phosphorylase A2 [42]. Dysfunctional HDL may promote the transfer of lipid hydroperoxides to apoB-containing lipoproteins and actually promote the VLDL and LDL oxidation required for atherosclerotic lesion formation [61]. Proinflammatory HDL-C has been associated with increased carotid intima-media thickness and an increased risk for adverse clinical outcomes [64]. HDL from patients with coronary artery disease does not prevent LDL oxidation [65]. On the other hand, raising the level of functional HDL particles by increasing their hepatic production or by HDL infusion results in atheroprotective effects [37].

HDL function can be measured *in vitro* [66]. The capacity of HDL-C to promote cholesterol efflux from macrophages is a recognized metric of HDL function and has recently been shown to have a strong inverse relationship with both carotid intima-media thickness and the presence of angiographically confirmed coronary artery disease, independent of HDL-C levels [67▪▪,68]. Determining HDL function may identify patients with normal or low HDL-C at high risk of CVD. Future therapeutic agents targeting HDL may be required to improve its functionality rather than simply raising its cholesterol cargo [36▪▪].

GENETICS

Common genetic variants associated with HDL-C, but not other lipoprotein traits, were not associated with MI in a recent Mendelian randomization study [60▪▪], suggesting that raising HDL-C pharmacologically will not necessarily translate into a reduced cardiovascular risk.

The antiatherogenic action of apoA-I is well established [69]. Mutations of *ApoA-I* could explain the functional heterogeneity of HDL particles [13]. Several genetic syndromes with reduced HDL-C and apoA-I are not associated with an increased risk of premature CVD [42]. Heterozygosity for the Milano or Paris mutations of the *ApoA-I* gene, despite HDL-C less than 0.5 mmol/l, does not confer early CVD risk [70]. Deficiency of plasma LCAT does not increase CVD risk despite HDL-C less than 0.4 mmol/l [71]. Tangier disease, caused by mutations in the ABCA1 transporter gene, in which HDL and apoA-I concentrations are virtually undetectable, is not associated with the increase in CVD expected from such an extreme phenotype [43]. Genetic deficiency of CETP increases HDL-C but would also be expected to impair RCT [72]. The mechanism by which HDL-C is increased is critical in determining whether it reduces atherosclerosis. Thus, increases in HDL-C through downregulation of SR-B1 by oestrogens and CETP activity modulation by CETP inhibitors may not reduce cardiovascular risk.

LIFESTYLE MODIFICATION

Lifestyle interventions have been shown to be effective in increasing HDL-C levels and decreasing cardiovascular risk [13]. Most current guidelines for the management of dyslipidaemia in patients with low HDL-C levels recommend a change in lifestyle, focussing on smoking cessation, regular exercise, weight loss, dietary modifications and modest alcohol consumption [73]. In patients already on statin therapy, these dietary and lifestyle changes can raise HDL-C levels by an additional 5–10% [74]. A Mediterranean-style diet rich in fruits and vegetables and high in polyunsaturated fats improves the anti-inflammatory properties of HDL, decreases LDL-C, increases HDL-C and reduces cardiovascular events [75]. A low glycaemic index carbohydrate diet has been shown to improve HDL-C, glycaemic control and inflammatory markers [76]. Weight loss achieved through exercise may be more effective at raising HDL-C concentrations than dieting [77], but it is again difficult to attribute the reduction in cardiovascular risk to increases in HDL-C alone [78]. Smoking cessation increases HDL-C by 3–5.6% without changes in LDL-C, total cholesterol or triglyceride levels. It also favours RCT, decreases CETP and can reduce oxidative stress by improving endothelial function [79]. Moderate alcohol consumption can increase HDL-C by as much as 12% [15]. It remains unclear how much this contributes to the reduced incidence of CVD with moderate consumption of alcohol [80,81]. Alcohol may interfere with CETP activity, reducing the transfer of cholesteryl ester from HDL [15].

CURRENT THERAPIES

Lipid-lowering treatment with hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) can achieve a relative risk reduction for CVD of 25–40% [73,82], which is accepted to be secondary to LDL-C reduction, but may also be related to increases in HDL-C or HDL functionality. Statins can increase HDL-C by 5–15% [83], with a comparable increase in apoA-I levels. A Study To Evaluate the Effect of Rosuvastatin on Regression of Coronary Atherosclerosis (ASTEROID) suggested that statin-induced reductions in atheroma volume are because of increases in HDL-C in addition to reductions in LDL-C. In the Lipoprotein and Coronary Atherosclerosis Study (LCAS), fluvastatin reduced angiographic progression in patients with low HDL-C levels significantly compared with those with high HDL-C levels [84]. Statins increase HDL-C by activating peroxisome proliferator-activated receptor-α (PPAR-α), increasing the expression of apoA-I and apoA-II [85]. They may also increase HDL-C by reducing hepatic CETP expression and, thus, CETP-dependent transfer of cholesterol from HDL to VLDL. As statins lower MPO levels, they may also preserve HDL function by creating a less inflamed environment [86]. There is also some evidence that PON1 activity may be augmented by atorvastatin [87].

If concomitant hypertriglyceridaemia is present, most guidelines recommend consideration of a

fibrate [1]. The hallmarks of fibrate therapy are a 30–50% reduction in plasma triglyceride levels and a 10–15% increase in HDL-C levels [88,89]. Despite this, the beneficial effect of fibrates on all-cause and cardiac mortality remains controversial [89]. Fibrates act as PPAR-α agonists. They also increase HDL-C by reducing the CETP-dependent transfer of cholesterol from HDL to apoB-containing lipoprotein particles. Their effect on lipid modification, however, does not appear to be consistent [31,90].

Bile acid sequestrants and cholesterol uptake inhibitors such as ezetimibe reduce cholesterol absorption. They have minimal effects on HDL-C. Omega-3 fatty acids reduce triglycerides in a dose-dependent manner, but their effect on cardiovascular events appears to be independent of HDL [91▪].

NIACIN (NICOTINIC ACID)

The interest in raising HDL-C has brought renewed focus on the oldest and most effective agent in increasing HDL-C. Niacin was first integrated in clinical practice in the 1960s, when it was the first lipid-modifying agent shown to have an effect on cardiovascular endpoints [18]. Despite promising results, high rates of intolerance and adverse effects have precluded its widespread use.

Niacin increases HDL-C by 23%, and reduces LDL-C by 20% and triglyceride levels by 40% [28]. In a recent meta-analysis of 11 randomized controlled trials, a therapeutic strategy including niacin was shown to reduce major coronary events by 25%, stroke by 26%, and all cardiovascular events by 27%. The cardiovascular benefit of niacin appeared to be greater in patients with diabetes and metabolic syndrome despite mild adverse effects on glucose levels [92].

Although a number of trials (Table 1) have demonstrated benefit from adding niacin to other lipid-lowering drugs [7,33,93–96], it is difficult to attribute the benefit of niacin to its HDL-C-raising properties as opposed to its ability to lower LDL-C. Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides and Impact on Global Health Outcomes (AIM-HIGH) showed no benefit from adding niacin to simvastatin [1] although it is considered to be underpowered and have other design flaws [97▪▪]. Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE), the largest study of niacin ever undertaken, showed no benefit of adding niacin and laropiprant (Tredaptive) to simvastatin, but did demonstrate significant side-effects [98▪▪]. Although HPS2-THRIVE had no cutoff for HDL-C and LDL-C was low at less than 2 mmol/l before randomization, no type of patient has yet been identified who benefitted from treatment [99].

FUTURE AGENTS

Interest in the inhibition of CETP was stimulated by a 1985 publication describing a Japanese population in which a low-activity genetic variant of CETP was associated with elevated HDL-C and relatively low rates of CVD [1].

The first oral CETP inhibitor to be assessed in major clinical trials was torcetrapib. Despite promising results in phase I and II studies, increases in HDL-C of 72% and decreases in LDL-C of 25% and triglycerides of 9%, the large-scale phase III Investigation of Lipid Level Management to Understand its Impact in Atherosclerosis Events (ILLUMINATE) trial was terminated early because the torcetrapib and atorvastatin group experienced increased all-cause mortality and cardiovascular events compared with recipients of atorvastatin alone [100]. This has been attributed to CETP-independent angiotensin–aldosterone activation with adverse effects on blood pressure. However, CVD rates were lower in torcetrapib-treated patients whose HDL-C increased above the median [101]. HDL isolated from torcetrapib-treated patients showed normal or enhanced promotion of cholesterol efflux [102].

The second CETP inhibitor to progress to phase III studies was dalcetrapib, which associates differently with CETP and has no angiotensin–aldosterone-stimulating or hypertensive effects. Disappointingly, the dal-OUTCOMES (efficacy and safety of dalcetrapib in patients with recent acute coronary syndrome) trial showed no effect from dalcetrapib on cardiovascular events, despite raising HDL-C by 25% [103▪▪]. Dalcetrapib was discontinued from clinical development in 2012 following the second prespecified interim analysis of primary end-point events (death from coronary heart disease, nonfatal myocardial infarction, ischaemic stroke, unstable angina or cardiac arrest with resuscitation).

A third agent, anacetrapib, has greater effects on HDL-C and LDL-C. Like dalcetrapib, it does not appear to cause hypertension or have any other 'off-target' effects. At present, there is no evidence to suggest any harm from complete CETP inhibition with anacetrapib (or its modulation with dalcetrapib) [104,105]. The phase III study Determining the Efficacy and tolerability of CETP Inhibition with Anacetrapib (DEFINE) examined its effect in high-risk patients already on statin therapy [104]. Anacetrapib reduced LDL-C by 40% and increased HDL-C

Table 1. Trials investigating the effect of adding niacin to other lipid-lowering agents

Trial	Year	Participants	Treatment arms	Study design	Outcome
Coronary Drug Project (CDP)	1975, 1986	8341 men with previous myocardial infarction	Oestrogen vs. dextrothyroxine vs. clofibrate vs. niacin vs. placebo	Randomized, placebo-controlled trial; endpoints recurrent nonfatal MI at 6 years and total mortality at 15 years	Niacin significantly decreased nonfatal recurrent myocardial infarction at 6 years and total mortality at 15 years
Cholesterol Lowering Atherosclerosis Study (CLAS)	1987	162 nonsmoking men aged 40–59 years with previous coronary bypass surgery	Colestipol + niacin vs. placebo	2-year randomized, placebo-controlled, angiographic trial	43% reduction in LDL-C; 37% elevation of HDL-C; atherosclerosis regression in 16.2% of colestipol–niacin treated vs. 2.4% placebo treated
Familial Atherosclerosis Treatment Study (FATS)	1990	146 men with apolipoprotein B levels >125 mg/dl, documented coronary artery disease, and family history of vascular disease	Lovastatin + colestipol vs. niacin + colestipol vs. placebo	2 1/2-year randomized, double-blind study; arteriography at baseline and after treatment	LDL-C decreased by 25% and HDL-C increased by 38%; coronary lesion progression seen in 39%
HDL-Atherosclerosis Treatment Study (HATS)	2001	160 patients with coronary disease, low HDL-C and normal LDL-C	Simvastatin + niacin vs. vitamins vs. simvastatin-niacin + antioxidants vs. placebo	3-year double-blind trial; endpoints arteriographic evidence of change in coronary stenosis and first cardiovascular event	Frequency of clinical endpoint 24% with placebo; 3% with simvastatin–niacin; 21% in antioxidant group; 14% in simvastatin–niacin + antioxidants
Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER)	2004 2006	167 patients with known CHD and HDL-C <1.16 mmol/l on statin (ARBITER 2) 130 patients completing ARBITER 2 (ARBITER 3)	Extended release niacin vs. placebo	Randomized placebo-controlled trials; primary endpoint change in carotid intima-media thickness at 12 (ARBITER 2) and 24 months (ARBITER 3)	HDL-C increased from 1.01 to 1.22 mmol/l; progression of atherosclerosis slowed by 68% (ARBITER 2); HDL-C increased from 1.02 to 1.26 mmol/l; additional regression of carotid intima-media thickness of 0.041 mm
Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol 6 – HDL and LDL Treatment Strategies in Atherosclerosis (ARBITER 6-HALTS)	2010	315 patients with coronary heart disease or equivalent with LDL-C <100 mg/dl and HDL-C <55 mg/dl while receiving stable statin treatment	Ezetimibe vs. extended-release niacin	14-month randomized study; primary endpoint change in carotid intima-media thickness	Terminated early on basis of prespecified interim analysis showing superiority of niacin over ezetimibe on change in carotid intima-media thickness

CHD, coronary heart disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MI, myocardial infarction.

Table 2. HDL-modifying drugs in development

Class	Mechanism of Action	Examples	Developer	Status
CETP inhibitors	Inhibit CETP to increase circulating HDL-C and lower LDL-C	Torcetrapib	Pfizer	Discontinued 2006
		Dalcetrapib	Hoffmann-La Roche	Discontinued 2012
		Evacetrapib	Lilly	Phase III trial ACCELERATE to present in 2015
		Anacetrapib	Merck	Phase III trial REVEAL to present in 2017
ApoA-I mimetics	Stimulate cholesterol efflux from macrophages via ABCAI and associate with HDL to induce preβ HDL formation	D-4F	Novartis	Phase II trials
Stimulator of apoA-I gene transcription	Increases apoA-I, preβ HDL, and HDL functionality	RVX-208	Resverlogix	Phase I trials
Liver receptor X (LXR) agonists	Activate RCT	DMHCA	Wyeth	Phase I trials
rHDL	Infusion of reconstituted or recombinant HDL particles	ApoA-I Milano–phospholipid complex	The Medicines Company	Phase II trials
		Wildtype apoA-I–phospholipid complex	Cerenis Therapeutics	Phase II trials

ABCA1, ATP-binding membrane cassette transporter A1; ACCELERATE, Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition With Evacetrapib in Patients at a High-Risk for Vascular Outcomes; apoA-1, apolipoprotein A-1; CETP, cholesteryl ester transfer protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RCT, reverse cholesterol transport; REVEAL, Randomized Evaluation of the Effects of Anacetrapib Through Lipid-modification.

by 138%. Cardiovascular outcomes are currently being investigated [91▪]. Although the development of dalcetrapib has been discontinued, a fourth CETP inhibitor, evacetrapib, is in development [106].

Drugs in development are shown in Table 2 [91▪,103▪▪,106–113].

CONCLUSION

Lowering LDL-C has been the primary focus in lipid modification for the prevention and treatment of atherosclerosis [2]. The ARBITER 6-HALTS study and others implied that raising HDL-C should be the next target to ameliorate the progression of CVD [32], but increases in HDL-C may not result in the cardiovascular benefit suggested by prospective observational studies [114]. To date, HDL-based therapy to reduce the residual risk of CVD remains a largely unfulfilled promise [115]. Although raising the level of functional HDL particles either by increasing their hepatic production or by HDL infusion appears promising [37], there is limited evidence that any of the clinical endpoints measured to date (plaque volume and inflammatory state of macrophages) are correlated with decreased events [116].

There is increasing evidence that HDL-C levels are not necessarily directly correlated to HDL particle function [117]. As we understand the structural components of HDL and exact roles of each component in HDL function, traditional lipid panel studies will likely be superseded by assays of HDL function or measurement of biomarkers associated with dysfunctional HDL [42]. There remains cautious optimism that CETP inhibition may be a viable option to reduce cardiovascular risk, but the concern that the inhibition of CETP might lead HDL to become dysfunctional has not yet been fully allayed.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ■ of special interest
- ■■ of outstanding interest

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2. A Review of Paradoxical HDL-C Responses to Fenofibrate, Illustrated by a Case Report

Abstract

High-density lipoprotein cholesterol (HDL-C) concentration is an independent risk factor for cardiovascular disease. Fibrates are widely used in the management of atherogenic dyslipidaemia, principally for their triglyceride-lowering and HDL-C–raising effects. Fibrates may cause paradoxical reductions in HDL-C. These reductions are usually modest, but significant reductions have been observed. The molecular mechanism for these paradoxical reductions remains unexplained despite advances in our understanding of lipid metabolism. This review considers possible mechanisms for this effect, illustrated by a patient with an observed reduction in HDL-C of 88% after introduction of fenofibrate.

High-density lipoprotein cholesterol (HDL-C) concentration is an independent risk factor for cardiovascular morbidity and mortality [127]. A 6% increase in HDL-C is associated with a 22% reduction in the incidence of myocardial infarction in epidemiological studies [128]. Increasing HDL-C has emerged as an attractive tool for preventing cardiovascular events [129], despite the failure of recent large randomized clinical trials to demonstrate an improvement in cardiovascular outcomes through the use of niacin and the cholesteryl ester transfer protein (CETP) inhibitors torcetrapib and dalcetrapib [130].

Although both Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study were negative outcome trials [52, 131], fenofibrate, a peroxisome proliferator-activated receptor-α (PPAR-α) agonist, is widely used in the management of atherogenic dyslipidaemia. Its triglyceride (TG)-lowering and HDL-C–raising effect is more pronounced when baseline HDL-C concentration is low [132]. Although effects vary depending on the population, a meta-analysis of 53 clinical studies enrolling 16,802 subjects indicated that fibrates can be expected to reduce plasma TG levels by 30% to 40% and raise HDL-C levels by 10% to 20% [51], but there is marked interindividual variability in response to drug action [133]. The effects of fenofibrate on HDL composition may vary in different lipoprotein phenotypes, and a pharmacogenetic association might predict response [134].

We report a 63-year-old woman with mixed dyslipidaemia and a family history of premature cardiovascular disease treated with atorvastatin for 10 years, who developed a paradoxical reduction in HDL-C after the introduction of fenofibrate. Her fasting lipid profile before addition of fenofibrate showed total cholesterol (TC) 6.4 mmol/L, low-density lipoprotein cholesterol (LDL-C) 3.4 mmol/L, TG 4.7 mmol/L, and HDL-C 0.85 mmol/L. Six months after its introduction, TC, LDL-C, and TG decreased to 4.3 mmol/L, 2.9 mmol/L, and 2.8 mmol/L, respectively, but HDL-C decreased to 0.1 mmol/L, giving a higher TC/HDL-C ratio. HDL-C remained suppressed during the subsequent 12 months, with measurements ranging from 0.18 to 0.57 mmol/L.

Several reports have suggested that fibrates, particularly fenofibrate, may cause paradoxical reductions in HDL-C levels in both diabetic and nondiabetic patients, with both elevated and lower pretreatment HDL-C levels, and when co-prescribed with statins or other medications, particularly thiazolidinediones [135]. Although this patient was treated with atorvastatin, her other medications (amlodipine, lansoprazole, and nitrofurantoin) have not been reported to be associated with paradoxical reductions. HDL-C lowering has also been reported to occur more frequently in females and in patients with lesser elevations of TG but higher uric acid levels than this patient [136]. Altered hepatic function has not been reported to affect response to fenofibrate, although our patient's liver function tests were normal throughout.

There is also disagreement as to the prevalence of this problem. Although Magee *et al*. reported a paradoxical decrease in HDL-C in 46% of patients prescribed fibrate therapy in their lipid clinic [137], a subsequent retrospective analysis of 581 patients reported that the incidence of paradoxical HDL-C reductions was relatively uncommon, occurring in 15.3% of patients [136]. In addition, the observed HDL-C reduction was of a modest degree, that is, around 10% [136]. This analysis was supported by evidence from a pooled database of 854 patients, where 16% had reductions from baseline in HDL-C at the study end point [129]. Reductions in HDL-C of up to 30% were observed in the placebo group, suggesting that reductions of <30% can be considered to reflect natural variability [129]. Only 3 patients exhibited reductions in HDL-C of >30% in magnitude [129]. Two of these had apolipoprotein (Apo) AI reductions commensurate with the observed HDL-C reductions [129]. In all patients with paradoxical decreases in HDL-C concentrations, there was a full recovery to within 10% of pretreatment concentrations within 3 months of cessation of fenofibrate treatment [137]. After discontinuation of fenofibrate, we observed a recovery in HDL-C from 0.57 to 0.97 mmol/L at 3 months, increasing to 1.15 mmol/L after 9 months.

Just as the molecular mechanism of the HDL increase after fenofibrate treatment remains relatively unclear, the mechanisms for these paradoxical decreases remain unresolved [137]. There may be a genetic element because several genetic polymorphisms have been related to the concentration and structure of HDL-C [137]. We have assessed key aspects of HDL metabolism in this patient while on fenofibrate and after its withdrawal (Table 2.1).

The effect of fibrates on triglycerides may be due to a combination of increased catabolism of plasma triglyceride-rich particles and inhibition of their secretion from the liver through increased hepatic β-oxidation and inhibition of *de novo* fatty acid synthesis [138]. This is achieved through induction of lipoprotein lipase (LPL) mediated lipolysis, increased fatty acid uptake, reduced TG production, increased removal of LDL particles, reduced neutral lipid exchange (between very low-density lipoprotein [VLDL] and HDL), increasing HDL production, and stimulation of reverse cholesterol transport (RCT) [133]. The elevation of HDL-C after fibrate treatment could thus be attributed partially to enhanced lipolysis of triglyceride-rich lipoproteins and redistribution of lipid components from these particles to HDL [139]. We observed an expected reduction in plasma TG from 4.7 to 2.69 mmol/L with the introduction of fenofibrate and a rise to 3.70 mmol/L after its discontinuation. Fenofibrate is believed to increase HDL-C by reducing the CETP-dependent transfer of cholesterol ester from HDL to apo B-containing lipoproteins [140]. Fenofibrate treatment has been shown to decrease CETP activity by up to 26% in subjects with combined hyperlipidaemia [141]. We observed an increase in CETP activity from 29.7 to 39.5 nmol/mL/h on stopping fenofibrate therapy, consistent with this study. Although decreased TG and increased HDL-C are linked by the action of CETP, an increase in ATP-binding

cassette transporter (ABCA1) activity may more directly contribute to raising HDL and preventing atherosclerosis development [142].

Activation of the transcription factor PPAR-α by fibrates lowers plasma TG levels by inducing the hepatic synthesis of LPL and by reducing the expression of apolipoprotein CIII, an inhibitor of LPL [133]. Up-regulation of PPAR-α is followed by an increase in plasma levels of HDL-C through increased hepatic synthesis of apo AI and apo AII [143]. After discontinuation of fenofibrate, we observed a paradoxical rise in Apo AI from 0.45 to 1.32 g/L, but Apo AII fell slightly (17.54 to 16.6 mg/dL). Fibrates also exert a negative and probably PPAR-independent effect on apo AI gene transcription mediated by sequence elements located in the apo AI basal promoter [144]. Apo AII may antagonize the PPAR-α activating activity of fenofibrate [136]. When fenofibrate is given to apo AII transgenic mice, there is a clear antagonism of PPAR-α activation, a 4-fold increase of non–HDL-C and quantitatively similar decreases in HDL-C with severe reductions of mouse plasma apo AI and apo AII [145]. Where apo AI has been measured in patients with paradoxical reductions in HDL-C after fenofibrate therapy, HDL-C decreases were accompanied by parallel decreases in apo AI concentrations, suggesting that the number of HDL-C particles are reduced through either decreased synthesis or increased catabolism of apo AI [146].

Guerin *et al*. noted that although HDL-C levels are raised by fenofibrate, no significant change is generally detected in the total plasma HDL mass [136]. They suggested that fenofibrate treatment leads to a rise in apo AI + AII particles at the expense of apo AI [147]. This would result in a rise of HDL2a, HDL3a, and HDL3b, with a more or less marked reduction of HDL2b and HDL3c [136]. Knopp and Walden reported that treatment with fenofibrate had no significant effect on HDL2 cholesterol levels, whereas HDL3 cholesterol levels increased 8% to 16% [148]. This was consistent with their finding that apo AII levels increased significantly (13% to 20%), whereas those of apo AI did not [148]. Accordingly, paradoxical decreases may be related to the different effects of fibrates in HDL-C subpopulations, reducing the number of large VLDL particles and shifting the HDL subpopulations toward smaller denser HDL3 particles [149]. We observed an increase in both HDL2 (0.39 to 0.62 mmol/L), and HDL3 (0.18 to 0.35 mmol/L) after discontinuation of fenofibrate.

Lipoprotein (a) (Lp(a)) comprises an emerging cardiovascular disease risk factor [150]. Fenofibrate is not expected to lower Lp(a) levels, and therapy has even been shown to raise Lp(a) levels [151], but we observed a significant increase in Lp(a) on discontinuing fenofibrate (91.55 to 112.19 mg/dL).

Lecithin cholesterol acyl transferase (LCAT) catalyses the transfer of an acyl group from TG to cholesterol to form cholesterol ester. Its inhibition should therefore lower HDL-C. Staels

et al. demonstrated that clofibrate, fenofibrate and gemfibrozil all provoke a lowering of liver LCAT messenger RNA levels, with fenofibrate causing the largest decrease [152]. Parallel to the changes in hepatic LCAT messenger RNA levels, plasma LCAT activity decreased in a dose-dependent fashion to nearly 50% of the control value at the highest dose tested [152]. Hepatic LCAT messenger RNA levels increased 3 days after cessation of fenofibrate administration and reached levels comparable to untreated controls 7 days after cessation of treatment [152]. We demonstrated an increase in plasma LCAT activity from 42.4 to 76.6 nmol/mL/h on discontinuing fenofibrate, consistent with these results.

Studies *in vitro* and in mice showed that fibrates increase the hepatic transcription of human *APO AI* and *APO AII*, decrease hepatic scavenger receptor class B type I protein, increase scavenger receptor class B type I–mediated and ABCA1-mediated cholesterol efflux from human macrophages, and increase plasma phospholipid transfer protein activity [153]. All of these effects may potentially contribute to the increase in HDL-C observed in humans [140]. It is assumed that a reduction in HDL-C is detrimental in that it infers a reduction in RCT [137]. However, it is possible that the function of RCT is maintained despite low HDL-C concentrations as seen in LCAT deficiency and Tangier disease [154]. We observed a small improvement in cholesterol efflux capacity from 22.9% to 26.7% and an associated improvement in serum paraoxonase from 45.3 to 49.3 nmol/mL/min after withdrawal of fenofibrate. This suggests detrimental effects on RCT and HDL antioxidant capacity resulting from fenofibrate treatment in this patient.

In a study by DeClercq *et al.*, fenofibrate paradoxically reduced the level of HDL-C in apo E–deficient mice by 24%, as compared with controls [155]. This is consistent with our observed increase in Apo E from 10.59 to 14.46 mg/dL on withdrawing fenofibrate, although the patient was found to be heterozygous for *Apo E2* on genotyping, associated with higher apo E levels. Apo E genetic variation significantly modulates the percentage reduction of apo B, triglyceride, and apo E levels in response to fibrates [156], and although *Apo E2* has been reported to enhance HDL-C response, statistically significant differences according to *Apo E* genotype have not been established [157].

Studies have implicated single nucleotide polymorphisms in genes such as *CYP7A1*, *PPARA*, and the *APO A1/C3/A4/A5* cluster in lipid response to fenofibrate [158]. The rs964184 locus near the *APO A1* gene has emerged as the most consistent predictor of lipid fenofibrate response, showing statistically significant associations for changes in HDL-C and TG, and approaching statistical significance for LDL-C [132]. The reported results do not support a role for this gene cluster in large-magnitude HDL-C reductions [129]. The HDL response to fenofibrate, being individually regulated may, in some individuals, potentially those carrying the 265C Apo AII polymorphism, be reduced or become outright negative, resulting in reduced HDL-C levels [136].

In this patient, with a paradoxical reduction in HDL-C after treatment with fenofibrate, we have assessed a number of factors of known or potential value in assessing cardiovascular risk. Although paradoxical reductions in HDL-C have been reported previously, the mechanism and effect on cardiovascular risk remains unclear. Our observation that the paradoxical reduction in HDL-C is accompanied by a parallel decrease in Apo AI, whereas other parameters responded as expected, suggests underlying decreased synthesis or increased catabolism of Apo AI.

Recent practice guidelines from the American College of Cardiology/American Heart Association state that non-statin therapies do not provide acceptable cardiovascular risk reduction benefits compared with their potential for adverse effects, but that fenofibrate may be considered alongside statin therapy [159]. Guidelines from the European Society of Cardiology/European Atherosclerosis Society also suggest the addition of fenofibrate to statin therapy for patients with higher TG and lower HDL-C levels, noting that trials of fibrate monotherapy have not demonstrated significant cardiovascular benefit [160]. Where fenofibrate is used, it is important that physicians are aware of the phenomenon described in this study. The importance of recognition of paradoxical reductions in HDL-C, and our observation that reductions are accompanied by reduced cholesterol efflux capacity, is underlined by the recent publication of results from the ACCORD Lipid Trial, which demonstrated an increased incidence of low HDL-C values in patients treated with fenofibrate [161].

	Before starting fenofibrate	6 months after starting fenofibrate	Before stopping fenofibrate	3 months after stopping fenofibrate
Cholesterol (mmol/l)	6.4	4.3	5.14	5.42
Triglycerides (mmol/l)	4.7	2.8	2.69	3.70
LDL-C (mmol/l)	3.4	2.9	3.34	2.76
HDL-C (mmol/l)	0.85	0.1	0.57	0.97
HDL2 (mmol/l)	-	-	0.39	0.62
HDL3 (mmol/l)	-	-	0.18	0.35
Apo AI (g/l)	-	-	0.45	1.32
Apo AII (mg/dl)	-	-	17.54	16.6
Apo B (g/l)	-	-	1.21	1.14
Apo E (mg/dl)	-	-	10.59	14.46
LCAT (nmol/ml/h)	-	-	42.4	76.6
CETP (nmol/ml/h)	-	-	29.7	39.5
PON1 (nmol/ml/min)	-	-	45.3	49.3
Lp(a) (mg/dl)	-	-	91.55	112.19
Efflux (ApoB depleted serum) (%)	-	-	22.9	26.7
Efflux (HDL) (%)	-	-	45	48.3
Uric Acid (mmol/l)	-	-	-	0.20
Weight (kg) / BMI (kg/m^2)	64.8 / 27.3	62.5 / 26.4	62.0 / 26.1	63.0 / 26.6

Table 2.1: Effect of fenofibrate on measured parameters. *LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; HDL2: HDL subfraction (density 1.063-1.125 g/ml); HDL3: HDL subfraction (density 1.125-1.21 g/ml); Apo AI: Apolipoprotein AI; Apo AII: Apolipoprotein AII; Apo B: Apolipoprotein B; Apo E: Apolipoprotein E; LCAT: Lecithin cholesteryl acyl transferase; CETP: Cholesterol ester transfer protein; PON1: Paraoxonase-1; Lp(a): Lipoprotein (a); BMI: Body Mass Index*

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A review of paradoxical HDL-C responses to fenofibrate, illustrated by a case report

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KEYWORDS:
High-density lipoprotein cholesterol;
High-density lipoprotein;
HDL;
Paradoxical reduction in high-density lipoprotein cholesterol;
Fenofibrate

Abstract: High-density lipoprotein cholesterol (HDL-C) concentration is an independent risk factor for cardiovascular disease. Fibrates are widely used in the management of atherogenic dyslipidemia, principally for their triglyceride-lowering and HDL-C–raising effects. Fibrates may cause paradoxical reductions in HDL-C. These reductions are usually modest, but significant reductions have been observed. The molecular mechanism for these paradoxical reductions remains unexplained despite advances in our understanding of lipid metabolism. This review considers possible mechanisms for this effect, illustrated by a patient with an observed reduction in HDL-C of 88% after introduction of fenofibrate.

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Although both Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study were negative outcome trials,[5,6] fenofibrate, a peroxisome proliferator-activated receptor-α (PPAR-α) agonist, is widely used in the management of atherogenic dyslipidemia. Its triglyceride (TG)-lowering and HDL-C–raising effect is more pronounced when baseline HDL-C concentration is low.[7] Although effects vary depending on the population, a meta-analysis of 53 clinical studies enrolling 16,802 subjects indicated that fibrates can be expected to reduce plasma TG levels by 30% to 40% and raise HDL-C levels by 10% to 20%,[8] but there is marked interindividual variability in response to drug action.[9] The effects of fenofibrate on HDL composition may vary in different lipoprotein phenotypes, and a pharmacogenetic association might predict response.[10]

We report a 63-year-old woman with mixed dyslipidemia and a family history of premature cardiovascular disease treated with atorvastatin for 10 years, who developed a paradoxical reduction in HDL-C after the introduction of fenofibrate. Her fasting lipid profile before addition of fenofibrate showed total cholesterol (TC) 6.4 mmol/L,

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low-density lipoprotein cholesterol (LDL-C) 3.4 mmol/L, TG 4.7 mmol/L, and HDL-C 0.85 mmol/L. Six months after its introduction, TC, LDL-C, and TG decreased to 4.3 mmol/L, 2.9 mmol/L, and 2.8 mmol/L, respectively, but HDL-C decreased to 0.1 mmol/L, giving a higher TC/HDL-C ratio. HDL-C remained suppressed during the subsequent 12 months, with measurements ranging from 0.18 to 0.57 mmol/L.

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Just as the molecular mechanism of the HDL increase after fenofibrate treatment remains relatively unclear, the mechanisms for these paradoxical decreases remain unresolved.[13] There may be a genetic element because several genetic polymorphisms have been related to the concentration and structure of HDL-C.[13] We have assessed key aspects of HDL metabolism in this patient while on fenofibrate and after its withdrawal (Table 1).

The effect of fibrates on triglycerides may be due to a combination of increased catabolism of plasma triglyceride-rich particles and inhibition of their secretion from the liver through increased hepatic β-oxidation and inhibition of de novo fatty acid synthesis.[14] This is achieved through induction of lipoprotein lipase (LPL) mediated lipolysis, increased fatty acid uptake, reduced TG production, increased removal of LDL particles, reduced neutral lipid exchange (between very low-density lipoprotein [VLDL] and HDL), increasing HDL production, and stimulation of reverse cholesterol transport (RCT).[9] The elevation of HDL-C after fibrate treatment could thus be attributed partially to enhanced lipolysis of triglyceride-rich lipoproteins and redistribution of lipid components from these particles to HDL.[15] We observed an expected reduction in plasma TG from 4.7 to 2.69 mmol/L with the introduction of fenofibrate and a rise to 3.70 mmol/L after its discontinuation. Fenofibrate is believed to increase HDL-C by reducing the CETP-dependent transfer of cholesterol ester from HDL to apo B-containing lipoproteins.[16] Fenofibrate treatment has been shown to decrease CETP activity by up to 26% in subjects with combined hyperlipidemia.[17] We observed an increase in CETP activity from 29.7 to 39.5 nmol/mL/h on stopping fenofibrate therapy, consistent with this study. Although decreased TG and increased HDL-C are linked by the action of CETP, an increase in ATP-binding cassette transporter (ABCA1) activity may more directly contribute to raising HDL and preventing atherosclerosis development.[18]

Activation of the transcription factor PPAR-α by fibrates lowers plasma TG levels by inducing the hepatic synthesis of LPL and by reducing the expression of apolipoprotein CIII, an inhibitor of LPL.[9] Up-regulation of PPAR-α is followed by an increase in plasma levels of HDL-C through increased hepatic synthesis of apo AI and apo AII.[19] After discontinuation of fenofibrate, we observed a paradoxical rise in Apo AI from 0.45 to 1.32 g/L, but Apo AII fell slightly (17.54 to 16.6 mg/dL). Fibrates also exert a negative and probably PPAR-independent effect on apo AI gene transcription mediated by sequence elements located in the apo AI basal promoter.[20] Apo AII may antagonize the PPARα activating activity of fenofibrate.[12] When fenofibrate is given to apo AII transgenic mice, there is a clear antagonism of PPARα activation, a 4-fold increase of non–HDL-C and quantitatively similar decreases in HDL-C with severe reductions of mouse plasma apo AI and apo AII.[21] Where apo AI has been measured in patients with paradoxical reductions in HDL-C after fenofibrate therapy, HDL-C decreases were accompanied by parallel decreases in apo AI concentrations, suggesting that the number of HDL-C particles are reduced through either decreased synthesis or increased catabolism of apo AI.[22]

Guerin et al. noted that although HDL-C levels are raised by fenofibrate, no significant change is generally detected in the total plasma HDL mass.[12] They suggested that fenofibrate treatment leads to a rise in apo AI + AII particles at the expense of apo AI.[23] This would result in a rise of HDL2a, HDL3a, and HDL3b, with a more or less marked reduction of HDL2b and HDL3c.[12] Knopp and Walden reported that treatment with fenofibrate had no significant effect on HDL2 cholesterol levels, whereas

Table 1 Effect of fenofibrate on measured parameters

	Before starting fenofibrate	6 months after starting fenofibrate	Before stopping fenofibrate	3 months after stopping fenofibrate
Cholesterol (mmol/L)	6.4	4.3	5.14	5.42
Triglycerides (mmol/L)	4.7	2.8	2.69	3.70
LDL-C (mmol/L)	3.4	2.9	3.34	2.76
HDL-C (mmol/L)	0.85	0.1	0.57	0.97
HDL2 (mmol/L)	-	-	0.39	0.62
HDL3 (mmol/L)	-	-	0.18	0.35
Apo AI (g/L)	-	-	0.45	1.32
Apo AII (mg/dL)	-	-	17.54	16.60
Apo B (g/L)	-	-	1.21	1.14
Apo E (mg/dL)	-	-	10.59	14.46
LCAT (nmol/mL/h)	-	-	42.4	76.6
CETP (nmol/mL/h)	-	-	29.7	39.5
PON1 (nmol/mL/min)	-	-	45.3	49.3
Lp(a) (mg/dL)	-	-	91.55	112.19
Efflux (ApoB depleted serum) (%)	-	-	22.9	26.7
Efflux (HDL) (%)	-	-	45.0	48.3
Uric acid (mmol/L)	-	-	-	0.20
Weight (kg)/BMI (kg/m^2)	64.8/27.3	62.5/26.4	62.0/26.1	63.0/26.6

Apo AI, apolipoprotein AI; Apo AII, apolipoprotein AII; Apo B, apolipoprotein B; Apo E, apolipoprotein E; BMI, body mass index; CETP, cholesteryl ester transfer protein; HDL-C, high-density lipoprotein cholesterol; HDL2, HDL subfraction (density 1.063-1.125 g/mL); HDL3, HDL subfraction (density 1.125-1.21 g/mL); LCAT, lecithin cholesteryl acyl transferase; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein (a); PON1, paraoxonase-1.

HDL3 cholesterol levels increased 8% to 16%.[24] This was consistent with their finding that apo AII levels increased significantly (13% to 20%), whereas those of apo AI did not.[24] Accordingly, paradoxical decreases may be related to the different effects of fibrates in HDL-C subpopulations, reducing the number of large VLDL particles and shifting the HDL subpopulations toward smaller denser HDL3 particles.[25] We observed an increase in both HDL2 (0.39 to 0.62 mmol/L), and HDL3 (0.18 to 0.35 mmol/L) after discontinuation of fenofibrate.

Lipoprotein (a) (Lp(a)) comprises an emerging cardiovascular disease risk factor.[26] Fenofibrate is not expected to lower Lp(a) levels, and therapy has even been shown to raise Lp(a) levels,[27] but we observed a significant increase in Lp(a) on discontinuing fenofibrate (91.55 to 112.19 mg/dL).

Lecithin cholesterol acyl transferase (LCAT) catalyses the transfer of an acyl group from TG to cholesterol to form cholesterol ester. Its inhibition should therefore lower HDL-C. Staels et al. demonstrated that clofibrate, fenofibrate and gemfibrozil all provoke a lowering of liver LCAT messenger RNA levels, with fenofibrate causing the largest decrease.[28] Parallel to the changes in hepatic LCAT messenger RNA levels, plasma LCAT activity decreased in a dose-dependent fashion to nearly 50% of the control value at the highest dose tested.[28] Hepatic LCAT messenger RNA levels increased 3 days after cessation of fenofibrate administration and reached levels comparable to untreated controls 7 days after cessation of treatment.[28] We demonstrated an increase in plasma LCAT activity from 42.4 to 76.6 nmol/mL/h on discontinuing fenofibrate, consistent with these results.

Studies in vitro and in mice showed that fibrates increase the hepatic transcription of human APOAI and APOAII, decrease hepatic scavenger receptor class B type I protein, increase scavenger receptor class B type I–mediated and ABCA1-mediated cholesterol efflux from human macrophages, and increase plasma phospholipid transfer protein activity.[29] All of these effects may potentially contribute to the increase in HDL-C observed in humans.[16] It is assumed that a reduction in HDL-C is detrimental in that it infers a reduction in RCT.[13] However, it is possible that the function of RCT is maintained despite low HDL-C concentrations as seen in LCAT deficiency and Tangier disease.[30] We observed a small improvement in cholesterol efflux capacity from 22.9% to 26.7% and an associated improvement in serum paraoxonase from 45.3 to 49.3 nmol/mL/min after withdrawal of fenofibrate. This suggests detrimental effects on RCT and HDL antioxidant capacity resulting from fenofibrate treatment in this patient.

In a study by DeClercq et al., fenofibrate paradoxically reduced the level of HDL-C in apo E–deficient mice by 24%, as compared with controls.[31] This is consistent with our observed increase in Apo E from 10.59 to 14.46 mg/dL on withdrawing fenofibrate, although the patient was found to be heterozygous for *Apo E2* on genotyping, associated with higher apo E levels. Apo E genetic variation significantly modulates the percentage reduction of apo B, triglyceride, and apo E levels in response to fibrates,[32] and although *Apo E2* has been reported to enhance HDL-C response, statistically significant differences according to *ApoE* genotype have not been established.[33]

Studies have implicated single nucleotide polymorphisms in genes such as *CYP7A1*, *PPARA*, and the *APOA1/C3/A4/A5* cluster in lipid response to fenofibrate.[34] The rs964184 locus near the *APOA1* gene has emerged as the most consistent predictor of lipid fenofibrate response, showing statistically significant associations for changes in HDL-C and TG, and approaching statistical significance for LDL-C.[7] The reported results do not support a role for this gene cluster in large-magnitude HDL-C reductions.[3] The HDL response to fenofibrate, being individually regulated may, in some individuals, potentially those carrying the 265C Apo AII polymorphism, be reduced or become outright negative, resulting in reduced HDL-C levels.[12]

In this patient, with a paradoxical reduction in HDL-C after treatment with fenofibrate, we have assessed a number of factors of known or potential value in assessing cardiovascular risk. Although paradoxical reductions in HDL-C have been reported previously, the mechanism and effect on cardiovascular risk remains unclear. Our observation that the paradoxical reduction in HDL-C is accompanied by a parallel decrease in Apo AI, whereas other parameters responded as expected, suggests underlying decreased synthesis or increased catabolism of Apo AI.

Recent practice guidelines from the American College of Cardiology/American Heart Association state that nonstatin therapies do not provide acceptable cardiovascular risk reduction benefits compared with their potential for adverse effects, but that fenofibrate may be considered alongside statin therapy.[35] Guidelines from the European Society of Cardiology/European Atherosclerosis Society also suggest the addition of fenofibrate to statin therapy for patients with higher TG and lower HDL-C levels, noting that trials of fibrate monotherapy have not demonstrated significant cardiovascular benefit.[36] Where fenofibrate is used, it is important that physicians are aware of the phenomenon described in this study. The importance of recognition of paradoxical reductions in HDL-C, and our observation that reductions are accompanied by reduced cholesterol efflux capacity, is underlined by the recent publication of results from the ACCORD Lipid Trial, which demonstrated an increased incidence of low HDL-C values in patients treated with fenofibrate.[37]

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3. Diabetes Dyslipidaemia

Abstract

Diabetes mellitus is associated with a considerably increased risk of premature atherosclerotic cardiovascular disease. Intensive glycaemic control has essentially failed to significantly improve cardiovascular outcomes in clinical trials. Dyslipidaemia is common in diabetes and there is strong evidence that cholesterol lowering improves cardiovascular outcomes, even in patients with apparently unremarkable lipid profiles. This review considers recent developments in the understanding of lipoprotein metabolism in diabetes and the implications of observed alterations in lipoproteins. I will also discuss the effect of medications commonly used in the management of diabetes on the lipid profile, the evidence for lifestyle and pharmaceutical interventions, concerns over the use of statin therapy, and national and international recommendations for the management of dyslipidaemia in patients with diabetes.

Background

Diabetes mellitus is associated with a considerably increased risk of premature atherosclerosis, particularly coronary heart disease (CHD) and peripheral arterial disease [14, 15]. Although more recent analyses have suggested a less marked effect, most authorities consider diabetes to confer at least a twofold excess risk, independently from other conventional risk factors [162, 163]. Even in people without diabetes, fasting blood glucose concentration and glycated haemoglobin (HbA1c) are associated with the risk of vascular disease [162, 164].

Early studies of cardiovascular mortality in type 1 diabetes (T1DM) suggested that risk only significantly increases after the development of nephropathy, which coincides with a marked deterioration of the lipid profile and blood pressure [165]. In patients with T1DM and proteinuria a 37-fold excess risk has been described, compared with a relative risk of 4.3 in patients without proteinuria [166]. Importantly this relative risk does not appear to be related to disease duration. More recent analyses have suggested that improved management of other risk factors can reduce the overall relative risk to 3.0 for women and 2.3 for men [167].

In type 2 diabetes (T2DM) an increased cardiovascular risk can exist for many years before the onset of biochemical hyperglycaemia. During this period obesity and insulin resistance are often present, associated with hypertension and dyslipidaemia, usually referred to as metabolic syndrome [168]. These risk factors may lead to the early development of CHD and may account for the increased incidence of diabetes in the period following a diagnosis of cardiovascular disease [169]. In keeping with this observation, about one in six patients with newly diagnosed T2DM enrolled in the United Kingdom Prospective Diabetes Study (UKPDS; Controlled-Trials.com identifier: ISRCTN75451837) had evidence of previous silent myocardial infarction [170].

However, neither the Diabetes Control and Complications Trial (DCCT; ClinicalTrials.gov identifier: NCT00360815) or the UKPDS (apart from 342 patients in the UKPDS metformin subgroup) showed a statistically significant reduction in CHD risk with more intensive glycaemic control [171, 172]. Both the Action in Diabetes and Vascular Disease (ADVANCE; ClinicalTrials.gov identifier: NCT00145925) and Veterans Affairs Diabetes (VADT; ClinicalTrials.gov identifier: NCT00032487) trials also failed to show the desired beneficial effects on cardiovascular outcomes from intensive glycaemic control [173, 174]. This apparent lack of effect was confirmed in the Outcome Reduction with Initial Glargine Intervention (ORIGIN; ClinicalTrials.gov identifier: NCT00069784) trial with insulin [175]. Reassuringly, prior intensive therapy was associated with long-term reductions in CHD during the observational follow-up studies of both the DCCT and UKPDS [176, 177], although

the Action to Control Cardiovascular Risk in Diabetes (ACCORD; ClinicalTrials.gov identifier: NCT00000620) study group reported previously unrecognized harm from intensive glucose lowering in patients with T2DM with established cardiovascular disease or additional cardiovascular risk factors [178].

Dyslipidaemia is a common feature of diabetes [50]. There is an association between atherosclerotic cardiovascular disease and serum cholesterol and triglyceride levels in both type 1 and type 2 diabetes [179, 180]. The risk of CHD is greater at any given level of serum cholesterol in patients with diabetes and its association with hypertriglyceridaemia is stronger than in the general population [181]. Importantly, there is strong and convincing evidence that cholesterol lowering therapy significantly reduces CHD in patients both with and without diabetes [182-184]. There also appears to be no threshold below which a further reduction in low-density lipoprotein (LDL) cholesterol might be beneficial [183, 184]. Evidence that statin treatment can substantially decrease the risk of atherosclerotic cardiovascular disease in T2DM was assimilated into guidelines for diabetes management soon after its publication [82, 185]. The same recommendations also embraced statin therapy in T1DM, although the clinical reasoning for this was different from that for T2DM [184].

Improved glycaemic control generally has favourable effects on lipoprotein levels in diabetes, with a reduction in cholesterol and triglyceride levels through decreased circulating very-low-density lipoprotein (VLDL) and by increased catabolism of LDL through reduced glycation and upregulation of LDL receptors [186, 187]. It is certainly possible that any cardiovascular benefit which might be derived from intensive glucose lowering is related to effects on lipoprotein metabolism rather than directly through altered glycaemia [188].

Dyslipidaemia and Atherosclerosis in Diabetes

The dyslipidaemia of T2DM is characterized by high triglyceride levels and decreased high-density lipoprotein (HDL) cholesterol, changes observed many years before the onset of clinically relevant hyperglycaemia [16, 168]. Recent evidence suggests that low HDL cholesterol is an independent risk factor not only for cardiovascular disease but also for the development of diabetes itself [189]. These changes, and the presence of small dense LDL particles, probably contribute to accelerated atherosclerosis even before diabetes is formally diagnosed [168, 190]. In T1DM, hypertriglyceridaemia may occur, but HDL cholesterol levels are often normal or even high unless glycaemic control is poor or nephropathy is present [16]. In addition, patients with diabetes show qualitative and kinetic abnormalities for all lipoproteins [191].

A number of factors may contribute to the alterations in lipid metabolism observed in patients with diabetes, including insulin deficiency or resistance, adipocytokines, and hyperglycaemia [191]. Many aspects of the pathophysiology and consequences of diabetes dyslipidaemia remain unclear, but the mechanism by which hypertriglyceridaemia arises is fairly well understood [192]. Insulin deficiency or resistance activates intracellular hormone-sensitive lipase which increases the release of non-esterified fatty acids (NEFA) from triglycerides stored in the more metabolically active centrally distributed adipose tissue [193]. High circulating levels of NEFA increase hepatic triglyceride production. Increased hepatic triglyceride synthesis is associated with increased secretion of apolipoprotein B (apo B) [194] (see Figure 3.1). Furthermore, the normal inhibitory effect of insulin on hepatic apo B production and triglyceride secretion in VLDL is lost, and the VLDL secreted is larger and more triglyceride-rich [195-197]. The tendency to hypertriglyceridaemia is further augmented by reduced VLDL catabolism [191]. In the liver the excess triglyceride synthesis may overcome the diminished capacity for VLDL secretion, resulting in nonalcoholic steatohepatitis. Lipoprotein lipase located on vascular endothelium largely determines the rate of removal of triglycerides from the circulation. In contrast to intracellular hormone-sensitive lipase this lipoprotein lipase may be downregulated in states of insulin resistance or deficiency [191]. This reduction in lipoprotein lipase activity also contributes to postprandial lipaemia [198].

It is essential to rectify the commonly held misconception that triglyceride concentration is a poor indicator of cardiovascular risk. There is a strong relationship between triglycerides and CHD in both type 1 and type 2 diabetes. Raised serum triglycerides herald the development of T2DM, particularly when associated with other features of metabolic syndrome or CHD, and once diabetes has developed they continue to predict CHD risk, often independently of other risk factors [179]. Triglycerides are positively correlated with cholesterol, obesity, glucose intolerance, cigarette smoking, and hyperuricaemia, and are negatively correlated with HDL cholesterol. When these factors are included in multivariate analysis, the element of risk attributable to triglycerides themselves appears less significant, but the risk associated with hypertriglyceridaemia is still substantial with fasting levels of 2.6–4.5 mmol/L associated with a twofold excess of CHD risk and levels of 4.5–9.0 mmol/L with up to a ninefold elevation [199, 200].

Triglyceride-rich lipoproteins (chylomicrons and VLDL) are not known to participate directly in atherogenesis, but they are central to the mechanism by which small dense LDL cholesterol is generated and HDL cholesterol levels are depressed in diabetes [14]. Levels of triglycerides need only exceed 1.5 mmol/l for small dense LDL to be generated [201]. Cholesteryl ester is transferred from other lipoproteins into the enlarged circulating pool of triglyceride-rich lipoproteins by cholesteryl ester transfer protein, and the rate of transfer appears to be increased in both type 1 and type 2 diabetes [202, 203], although this

requires confirmation. There is transfer of triglyceride in the opposite direction such that cholesteryl ester-depleted HDL and LDL become triglyceride-rich. The subsequent removal of this triglyceride by hepatic lipase results in smaller, denser HDL and LDL particles.

Levels of small dense LDL are thus increased, and the apparently relatively undisturbed cholesterol and apo B levels observed in many patients with diabetes may thus hide a major atherogenic change [18-20]. Circulating LDL is not believed to participate directly in atherogenesis, but must first undergo structural modification to allow its apo B to act as a ligand for the scavenger receptors of monocyte macrophages in the arterial wall, triggering foam cell formation [204]. Small dense LDL is more susceptible to chemical modifications such as oxidation and glycation [205, 206]. Indeed, circulating levels of glycated apo B are strongly determined by the concentration of small dense LDL (Figure 3.2) [205]. The proportion of glycated apo B is doubled even in reasonably well-controlled diabetes [187]. Glycated LDL may be more susceptible to oxidation or itself represent an atherogenic modification [207, 208]. Glycation of LDL also decreases its LDL receptor-mediated catabolism [209]. Statin treatment is probably effective because, in addition to lowering intermediate density lipoprotein (IDL) and LDL cholesterol, it decreases circulating levels of small dense, oxidised and glycated LDL [205]. Whether oxidised and glycated LDL levels are increased in T1DM patients has never been reliably reported.

In patients with T1DM with good glycaemic control, insulin upregulates lipoprotein lipase, increasing the production of small HDL particles [210, 211], frequently to higher than normal HDL cholesterol levels [212]. HDL cholesterol in diabetes has thus not arisen physiologically; HDL dysfunction is suggested by the observation that high HDL cholesterol levels do not always protect against CHD [11, 130]. HDL may be dysfunctional in its capacity to protect LDL against atherogenic modification. Reductions in the antioxidative and anti-inflammatory effects of HDL have been reported in patients with diabetes, alongside an impaired ability of HDL to counteract the inhibition of endothelium-dependent vasorelaxation by oxidized LDL [192, 213]. The role of HDL in protecting pancreatic beta cells against apoptosis is an important emerging area of research [214]. Glycation has recently been shown to reduce the sphingosine-1-phosphate content of HDL, reducing its ability to activate protective intracellular survival pathways during oxidative stress [215].

With the development of nephropathy, increased catabolism of smaller HDL particles likely combines with increased cholesteryl ester transfer to lower HDL cholesterol levels [165, 216]. Glycation of HDL further enhances its catabolism [217]. Diabetes thus leads to impaired reverse cholesterol transport through both reduced HDL concentrations and HDL dysfunction [194]. Low HDL cholesterol appears to be of greater importance in patients at high cardiometabolic risk and is amongst the factors that currently favour a decision to

further reduce LDL cholesterol levels [50]. Lipoprotein(a) also increases in nephropathy [218, 219].

Clinical Investigations

The apparently normal serum cholesterol concentrations observed in both type 1 and type 2 diabetes led to the widespread erroneous belief that glycaemia alone might explain the observed high CHD rates. However, the relatively normal cholesterol levels hide an atherogenic lipid profile, with increased intermediate-density lipoprotein and small dense LDL, and dysfunctional HDL [207-209].

Each LDL particle, regardless of its density or cholesterol content, contains only a single tightly bound molecule of apo B. The often "normal" level of LDL cholesterol seen in many patients with diabetes actually disguises an increased particle number (higher apo B) and that clearance of small dense particles is slower [50]. Thus, measurement of the serum concentration of apo B provides a more discriminating index of atherogenic risk or therapeutic response than LDL cholesterol [220]. While intensive therapy in the DCCT did not significantly affect LDL and HDL cholesterol levels, it was associated with decreased apo B (and lipoprotein(a)) and with favourable alterations in lipoprotein subclasses that are not revealed by the standard lipid profile, but have implications for the evolution of both microvascular and macrovascular complications [221].

The use of LDL cholesterol in diabetes also underestimates the atherogenic contribution of triglyceride-rich particles so non-HDL cholesterol is a better measure of atherogenicity in diabetes [6]. The introduction of non-HDL cholesterol appears to have obviated the need to introduce apo B measurements more widely in patient management.

Diet and Weight Management

Lifestyle modifications are the first-line intervention in the management of diabetes dyslipidaemia, and include weight loss, dietary modification, and aerobic exercise [194]. Obesity increases insulin resistance and is associated with increased triglycerides and LDL cholesterol and decreased HDL cholesterol [14]. Weight loss is known to be associated with improvements in lipids and other cardiovascular risk factors including the incidence of T2DM [222, 223] and should therefore be encouraged in overweight patients with diabetes. To achieve sustained weight loss, caloric restriction remains the key and even modest degrees of weight loss are associated with an improvement in glycaemic control, HbA1c, and lipid profile [224].

Increased physical activity may provide some small adjunct to the effect of dietary restriction, but is unlikely to be successful on its own. Reduced fat intake, particularly of saturated fat, should also be encouraged [225]. The American Diabetes Association (ADA) recommends a diet low in trans fat, saturated fat, and cholesterol [226]. In patients without a marked increase in serum triglycerides but who are not obese, some substitution of saturated fat can be made with unrefined carbohydrate foods and some with oleic, linoleic, or omega-3 fish oils [227].

Dietary interventions, while considered first-line treatment for all patients with diabetes, have not been successful in demonstrating a mortality benefit, even with prolonged follow-up [228].

The gastrointestinal lipase inhibitor orlistat causes fat malabsorption and should be taken close to meals. The patient must adhere to a low fat diet or they will experience steatorrhoea. There is often early benefit, but then weight loss levels out, likely as the patient learns to omit it if they plan to consume a fatty meal. Nonetheless any weight loss achieved can improve cardiovascular risk factors [229]. Orlistat has beneficial effects on serum total and LDL cholesterol levels which are greater than might be explained by weight loss alone [230].

Surgical management of obesity is much more effective than medical treatment [222]. Weight loss after bariatric surgery is also associated with beneficial glycaemic effects in diabetes, including achievement of near normal glycaemia without medication or reduced medications [231]. We recently reported favourable changes in HDL functionality and composition, systemic inflammation, and perivascular adipose properties and anticontractile function [232-234].

Effects of Hypoglycaemic Agents on Lipoproteins

Diabetes dyslipidaemia can be partly corrected by insulin treatment and improved blood glucose control [235]. Insulin therapy increases HDL cholesterol and reduces circulating triglyceride levels, particularly in patients with poor glycaemic control [236]. Metformin decreases serum triglycerides and improves insulin resistance but is often overlooked as a lipid-lowering agent and is generally considered only as a hypoglycaemic agent in the management of diabetes [237]. Other drugs used in the management of diabetes may also have unintended positive and negative effects on lipoproteins (see Table 3.1). Of particular interest is the small increase in LDL cholesterol observed following treatment with sodium-glucose cotransporter 2 (SGLT2) inhibitors, suggesting that the recently reported significant improvement in cardiovascular outcomes with empagliflozin is unrelated to effects on dyslipidaemia [238].

Lipid-Lowering Drugs

The discovery of statins was a key advance in cardiovascular medicine. Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in cholesterol biosynthesis. There is a wealth of clinical trial evidence that lowering serum cholesterol with statins decreases the risk of CHD [35, 182]. These trials have demonstrated that statins decrease the risk of both CHD and stroke in people with and without pre-existing cardiovascular disease [182, 239]. It would appear from such trials that the relative risk reduction achieved with statin treatment is similar in patients with diabetes to that in other people. However, the number needed to treat to prevent one event (NNT) will be lower in patients with diabetes compared to those without diabetes but apparently similar lipid profiles [21].

The case for secondary prevention with statin therapy in diabetes is accepted, but whether all patients with diabetes should be considered for statin treatment has been more contentious. In the Heart Protection Study (Controlled-Trials.com identifier: ISRCTN48489393) the 2912 patients with diabetes without pre-existing vascular disease randomised to receive simvastatin showed a significant reduction in cardiovascular end-points compared to those allocated to placebo [240]. Similarly, in the Collaborative Atorvastatin Diabetes Study (CARDS; ClinicalTrials.gov identifier: NCT00327418), where the mean pretreatment LDL cholesterol was 3.0 mmol/L (<2.5 mmol/L in 25% of patients), the effect of active intervention with atorvastatin was so favourable that the study was stopped early [182]. In both studies there did not appear to be any threshold below which statin therapy ceased to be beneficial. A meta-analysis of 18,686 people with diabetes from 14 randomized trials (1466 with type 1 and 17,220 with type 2) demonstrated a 9% reduction in all-cause mortality for every 1 mmol/L reduction in LDL cholesterol [184]. The effects of statin therapy were similar irrespective of baseline characteristics and prior history of vascular disease. Although the majority of participants in these trials had T2DM, the reduction in major vascular events was also statistically significant in people with T1DM.

The risk factor reduction brought about by statins occurs in a dose-dependent fashion, with higher dose statins associated with a greater lowering of cardiovascular events [241]. It should also be noted that in diabetes, in contrast to patients without diabetes, statins do not stop the progression of carotid intima media thickness or intravascular ultrasound-measured atheroma volume at typical doses, implying that high doses may be necessary to prevent atheroma progression [242]. One in seven patients with diabetes treated with statins still goes on to suffer a cardiovascular event over 5 years [184].

Clinical trial evidence therefore provides unequivocal evidence to support prescribing statins for both primary and secondary prevention in diabetes, but the populations studied may not be representative of younger patients or those with advanced renal disease. This requires further exploration and thus the exercise of clinical judgment in prescribing.

Interestingly, statin treatment is associated with a slight increase in the incidence of T2DM. A meta-analysis showed 4 years of statin treatment in 255 patients would lead to one extra case of T2DM [243]. However, this risk is low both in absolute terms and when compared with the expected cardiovascular benefit from reducing LDL cholesterol. In the same period 5.4 vascular events would be avoided in these 255 patients [243]. Clinical practice in patients with existing cardiovascular disease or moderate or high cardiovascular risk should not change. Effects of lipid-modifying agents on lipoproteins and glucose metabolism are shown in Table 3.2.

Ezetimibe blocks the absorption of dietary cholesterol and the reabsorption of cholesterol entering the small intestine in bile, the latter accounting for most of its LDL cholesterol lowering. Ezetimibe is concentrated in the cells of the intestinal brush border, where it inhibits cholesterol absorption by a process which involves binding to Niemann-Pick C1-Like 1 [244]. It remains a second-line option for LDL cholesterol lowering in diabetes and has its greatest clinical utility as an adjunct to statin therapy.

The bile acid sequestrant colesevelam has been shown to reduce HbA1c in addition to reductions in total cholesterol, LDL, and non-HDL cholesterol levels [245]. Cholestyramine may be more effective in lowering LDL cholesterol, but both agents may increase triglycerides, and neither is particularly well tolerated [246].

Many novel therapies are in development to reduce LDL cholesterol, but none has been studied specifically in patients with diabetes [100]. In particular, proprotein convertase subtilisin/kexin 9 (PCSK9) inhibitors have emerged as medications showing significant reductions in LDL, with recent data suggesting similar effects on lipoproteins in patients with T2DM to those seen in patients without diabetes [247]. An additional potentially beneficial effect on postprandial hypertriglyceridaemia is suggested by novel data on PCSK9 regulation of intestinal lipoprotein assembly and secretion [248]. Ongoing cardiovascular outcome trials will inform the use of PCSK9 inhibitors in diabetes.

The ACCORD study showed an increased number of cardiovascular events in patients with diabetes and persistently low HDL cholesterol and high triglyceride levels, despite a mean LDL cholesterol below 2.1 mmol/L [52]. Fibrates act as peroxisome proliferator-activated receptor (PPAR)-α agonists to reduce triglycerides and modestly increase HDL cholesterol but also affect multiple pathways linked to the retinoid-X receptor [249]. To date no

decrease in cardiovascular outcomes has been convincingly demonstrated in clinical trials [51]. In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD; Controlled-Trials.com identifier: ISRCTN64783481) study 9795 people with T2DM were randomised to receive micronized fenofibrate or placebo [131]. By the end of the trial statin treatment became a major confounding factor, but the primary end-point (fatal CHD and non-fatal myocardial infarction) decreased by 11% on fenofibrate compared to placebo. Although pretreatment serum triglyceride levels appeared to have no influence on the relative decrease in cardiovascular events, the overall effect was not significant, and the FIELD study did not establish a firm place for fibrate drugs in the management of diabetes dyslipidaemia. More recently the ACCORD-LIPID trial reported no cardiovascular benefit from the addition of fenofibrate to simvastatin in patients with T2DM [52]. However, there did appear to be a beneficial effect on CHD outcomes in patients with triglycerides above 2.4 mmol/L and HDL cholesterol below 0.79 mmol/L [52]. There may therefore be a role for fibrates as adjunctive therapy in patients with diabetes and persistently elevated triglycerides. However, fibrates, particularly fenofibrate, may cause paradoxical reductions in HDL cholesterol levels and it is important that clinicians are aware of this phenomenon [250].

Most hypertriglyceridaemia in diabetes is mild to moderate and statins remain the drug of first choice even in patients with mixed dyslipidaemias, but in patients with genetic susceptibility severe hypertriglyceridaemia may develop, with attendant risk of pancreatitis [251]. When triglycerides are above 11 mmol/L a fibrate should be considered first to reduce triglycerides and the risk of pancreatitis.

Purified omega-3 fatty acids can lower triglycerides as much as fibrates, but they have little effect on HDL or LDL cholesterol [252]. Omacor® (Abbott Healthcare, Abbott Park, IL, USA) contains 90% omega-3 fatty acid ethyl esters (mostly eicosopentaenoate or docosahexaenoate) and in combination with a statin can decrease triglycerides by a further 30%, most probably by inhibition of hepatic triglyceride synthesis [253]. Despite evidence that omega-3 fatty acids stabilise atheromatous plaques and reports of beneficial hypotensive and antithrombotic effects, a recent trial of omega-3 fatty acids in patients with metabolic syndrome or T2DM disappointingly found no effect on CHD risk [254, 255].

Another future therapeutic option may be the dual PPAR-α/γ agonist saroglitazar, which was approved in India in 2013 and has been shown to significantly reduce plasma triglyceride, total cholesterol, non-HDL cholesterol, and VLDL cholesterol, and HbA1c and fasting glucose levels [256].

National and International Recommendations for the Management of Hyperlipidaemia

The role of diabetes in risk assessment processes is more controversial than the simple statement often applied in guidelines that diabetes is a cardiovascular disease-risk equivalent.

Risk in T1DM is strongly related to glycaemic control, nephropathy, and hypertension and can be significantly increased compared with normoglycaemic subjects [257]. In a large series of patients with T1DM 10-year CVD mortality exceeded 5% between the ages of 30 and 40 and this is likely to represent a combined 10-year CVD morbidity and mortality of 15–20% [14, 15, 258].

Risk in T2DM is still widely considered to be increased two- to fourfold [162, 259]. While features such as the presence of nephropathy or retinopathy identify higher risk groups, the use of other biomarkers of risk and likely need for enhanced treatment are often not appreciated. The presence of microalbuminuria for example is a risk factor for CHD even at low levels and its severity is also predictive of future events [260].

Most clinical guidelines recommend tight control of dyslipidaemia, especially in high-risk patients [261, 262]. Importantly, lipid targets are easier to achieve than blood pressure or glycaemia targets and target LDL cholesterol levels as low as 1.8 mmol/L are increasingly recommended in patients with established CHD [263, 264].

A recent position statement from the ADA recommends a screening lipid profile at the time of diagnosis, at age 40 years, and periodically thereafter [265]. Treatment recommendations beyond lifestyle modification and optimization of glycaemic control are for the use of high-intensity statin therapy (e.g. atorvastatin 40–80 mg or rosuvastatin 20–40 mg) in patients of all ages with overt CHD, and those aged 40–75 years with additional risk factors, and moderate intensity statin therapy (e.g. atorvastatin 10–20 mg or simvastatin 20–40 mg) for patients aged over 40 years without additional risk factors. Clinical judgment should guide the use of moderate or high-intensity statin therapy in patients younger than 40 years or older than 75 years with additional risk factors [265].

The most recent American Heart Association/American College of Cardiology guidelines for cholesterol management appear less interventional, recommending statin treatment only for patients with diabetes with clinical atherosclerotic cardiovascular disease or if aged 40–75 years [159]. There is also a modest recommendation for additional treatment in persons with statin intolerance or an inadequate response, with an emphasis on clinical judgment. The European Society of Cardiology suggests that ezetimibe be added after intensification of statin therapy in diabetes [160].

The National Institute for Health and Care Excellence also recently updated its guidance and now advises clinicians to offer statin treatment for primary prevention to adults with T1DM who are over 40 years, have had diabetes for more than 10 years, or have established nephropathy or other cardiovascular risk factors [266]. These guidelines also recommend statin therapy for primary prevention in T2DM if the 10-year risk of developing cardiovascular disease is estimated to be greater than 10% using the QRISK2 assessment tool [266], a higher level of estimated risk than the 7.5% in the next 10 years recently suggested in the USA [159].

Three important caveats must be considered with regard to risk estimation in diabetes. Firstly, the many rival risk engines which have been developed are all based on survivor populations and inevitably omit people who have already sustained cardiovascular events before the observed cohort was assembled. As such, they will underestimate risk. The Renfrew and Paisley Study [267] is important because it observed people from a random population sample, who developed diabetes during a long period of follow-up. It revealed that in the first 5 years after the onset of T2DM cardiovascular risk increases such that in the subsequent 5 years it reaches that of non-diabetic people with established cardiovascular disease, and in the 5 years after that rises further. Secondly, very few, if any typical T2DM patients will not have achieved at least a 7.5 or 10% 10-year CVD risk by middle age. This means that it will be younger people who, as the result of risk assessment, will be denied statin treatment, yet the risk estimation is at its most inaccurate in younger people with diabetes, because they were not well represented in the populations from which data to design the risk engines were derived. We know that in people who are so insulin-resistant as to develop T2DM in youth either because of extreme obesity or a genetic cause, the lifetime risk of cardiovascular disease is astronomic [268]. So any attempt to withhold treatment from them seems perverse. Thirdly, it is often seen as an advantage that the multivariate equations on which these risk engines are based have as variables, not only the mutable risk factors (lipids, blood pressure, and smoking), but also age, sex, obesity indices, family history, HbA1c, and so on. Additional risk factors might modestly improve the proportion of observed cardiovascular disease risk explained by the model, but each additional risk factor will erode the contribution of risk factors already in the equation. In biological terms this is because many risk factors such as obesity, age, and family history operate through cholesterol or blood pressure; and in mathematical terms because the multiple correlation coefficient can never exceed unity so each additional risk factor added to the model will reduce the contribution of others. This leads to an apparent underestimation of the contribution, say of LDL cholesterol, to risk. We know from meta-analysis of randomised clinical trials which shows a 22% decrease in cardiovascular disease risk for each 1 mmol/l that decreasing LDL cholesterol has a substantially greater effect than predicted in currently recommended multivariate risk prediction models [91]. Recent work suggests that much of the excess cardiovascular disease incidence in diabetes is

because of inadequate treatment of lipids and blood pressure [269]. One of the most disconcerting aspects of diabetes care to emerge recently is that the incidence of cardiovascular deaths among young women with T1DM now exceeds that of men [270]. These young women are precisely the group in whom statins are most often withheld [271].

In conclusion, statin treatment should effectively be considered for all people with diabetes aged over 40 years or younger if additional cardiovascular risk factors are present, a position recently supported by the Joint British Societies [261].

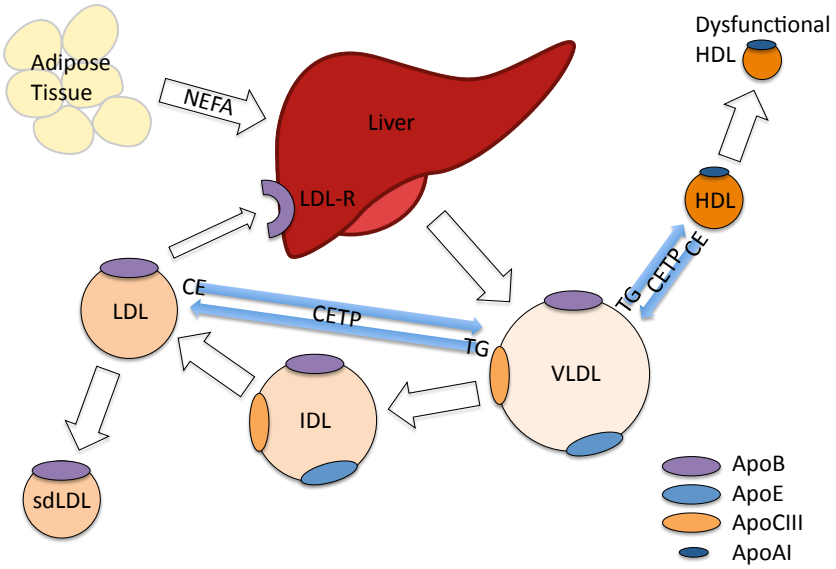

Figure 3.1: Qualitative changes in lipoproteins in diabetes. *Apo AI: apolipoprotein AI; Apo B: apolipoprotein B; ApoCIII: apolipoprotein CIII; Apo E: apolipoprotein E; CE: cholesteryl esters; CETP: cholesteryl ester transfer protein; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; LDL-R: low-density lipoprotein receptor; NEFA: non-esterified fatty acids; sdLDL: small dense low-density lipoprotein; TG: triglycerides; VLDL: very-low-density lipoprotein*

Drug	Total cholesterol	LDL cholesterol	HDL cholesterol	Triglycerides	References
Metformin	↓↔	↓	↔↑	↓↔	[272]
Gliclazide	↓	↔	↔	↓	[273, 274]
Glimepiride	↔	↔	↔↑	↔	[273, 275]
Pioglitazone	↑	↔	↑	↓	[276, 277]
Sitagliptin	↔	↔	↔↑	↔	[278, 279]
Saxagliptin	↔	↔	↔	↔	[274, 280]
Vildagliptin	↔	↔	↔↑	↔	[281]
Linagliptin	↔	↔	↔	↔	[282]
Dapagliflozin	↔↑	↔↑	↔↑	↓↔	[283, 284]
Canagliflozin	↑	↑	↑	↑	[279, 285]
Empagliflozin	↔↑	↔↑	↔↑	↔	[238, 286]
Exenatide	↓↔	↔↑	↔↑	↓	[277, 287]
Liraglutide	↔	↔	↔	↓	[288, 289]

Table 3.1: Effects of hypoglycaemic agents on lipoproteins. ↑ Increased; ↔ No change; ↓ Decreased. *HDL* High-density lipoprotein, *LDL* Low-density lipoprotein.

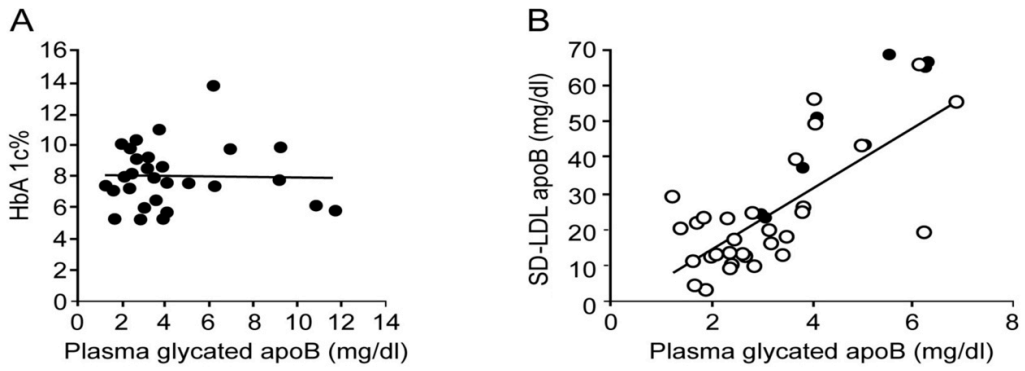

Figure 3.2: Plasma concentration of glycated apo B in type 2 diabetes plotted as a function of serum HbA1c (A) (*R* = 0.01; *P* = NS) and SD-LDL-apo B (B) (*R* = 0.74; *P* < 0.01). *Glycated apoB: glycated apolipoprotein B; HbA1c: glycated haemoglobin; SD-LDL apoB: small-dense low-density lipoprotein apolipoprotein B100*

Drug	TC	LDL-C	HDL-C	TG	apoB	Non-HDL-C	Lp(a)	Effect on glycaemia*
Statins	↓	↓	↔↑	↓↔	↓	↓	↔	↑
Ezetimibe	↓	↓	↔	↔	↓	↓	↔	↔
Fibrates	↓	↓	↓	↓	↓	↓	↔	↔↓
Colesevelam	↓	↓	↔	↔↑	↓	↓	↔	↓
PCSK9 monoclonal antibodies	↓	↓	↔	↓↔	↓	↓	↓	↔
CETP inhibitors**	↓↔	↓↔	↑	↓↔	↓↔	↓	↓[¥]	↓**
Purified omega-3 fatty acids	↔	↔	↔↑	↓	↔	↔	↔	↔

Table 3.2: Effects of Lipid Modifying Agents on Lipoproteins and glucose metabolism. ↓, decrease; ↑, increase; ↔, no change. *ApoB, apolipoprotein B100; CETP, cholesteryl ester transfer protein; LDL-C, low-density lipoprotein cholesterol; non-HDL-C; non-high density lipoprotein cholesterol; PCSK9, Proprotein convertase subtilisin/kexin type 9; TC, total cholesterol; TG, triglycerides;*

* Limited data available for other classes compared to statins. **CETP inhibitor anacetrapib is reported to improve insulin sensitivity and reduceHbA1c. [¥] Anacetrapib and TA-8995 reduce Lp(a).

Diabetes Dyslipidemia.

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REVIEW

Diabetes Dyslipidemia

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ABSTRACT

Diabetes mellitus is associated with a considerably increased risk of premature atherosclerotic cardiovascular disease. Intensive glycemic control has essentially failed to significantly improve cardiovascular outcomes in clinical trials. Dyslipidemia is common in diabetes and there is strong evidence that cholesterol lowering improves cardiovascular outcomes, even in patients with apparently unremarkable lipid profiles. Here, the authors review the pathophysiology and implications of the alterations in lipoproteins observed in both type 1 and type 2 diabetes, the effect of medications commonly used in the management of diabetes on the lipid profile, the evidence for lifestyle and pharmaceutical interventions, and national and international recommendations for the management of dyslipidemia in patients with diabetes.

Keywords: Cardiovascular risk; Diabetes; Dyslipidemia; Lipoproteins; Low density lipoprotein cholesterol

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BACKGROUND

Diabetes mellitus is associated with a considerably increased risk of premature atherosclerosis, particularly coronary heart disease (CHD) and peripheral arterial disease [1, 2]. Although more recent analyses have suggested a less marked effect, most authorities consider diabetes to confer at least a twofold excess risk, independently from other conventional risk factors [3, 4]. Even in people without diabetes, fasting blood glucose concentration and glycated hemoglobin (HbA1c) are associated with the risk of vascular disease [3, 5].

Early studies of cardiovascular mortality in type 1 diabetes suggested that risk only significantly increases after the development of nephropathy, which coincides with a marked deterioration of the lipid profile and blood pressure [6]. In patients with type 1 diabetes and proteinuria a 37-fold excess risk has been described, compared with a relative risk of 4.3 in patients without proteinuria [7]. Importantly this relative risk does not appear to be related to disease duration. More recent analyses have suggested that improved management of other risk factors can reduce the overall relative risk to 3.0 for women and 2.3 for men [8].

In type 2 diabetes an increased cardiovascular risk often exists for many years before the onset of biochemical hyperglycemia. During this period obesity and insulin resistance are often present, associated with hypertension and dyslipidemia, usually referred to as metabolic syndrome [9]. These risk factors may lead to the early development of CHD and may account for the increased incidence of diabetes in the period following a diagnosis of cardiovascular disease [10]. In keeping with this observation, about one in six patients with newly diagnosed type 2 diabetes enrolled in the United Kingdom Prospective Diabetes Study (UKPDS; Controlled-Trials.com identifier: ISRCTN75451837) had evidence of previous silent myocardial infarction [11].

However, neither the Diabetes Control and Complications Trial (DCCT; ClinicalTrials.gov identifier: NCT00360815) or the UKPDS (apart from 342 patients in the UKPDS metformin subgroup) showed a statistically significant reduction in CHD risk with more intensive glycemic control [12, 13]. Both the Action in Diabetes and Vascular Disease (ADVANCE; ClinicalTrials.gov identifier: NCT00145925) and Veterans Affairs Diabetes (VADT; ClinicalTrials.gov identifier: NCT00032487) trials also failed to show the desired beneficial effects on cardiovascular outcomes from intensive glycemic control [14, 15]. This apparent lack of effect was confirmed in the Outcome Reduction with Initial Glargine Intervention (ORIGIN; ClinicalTrials.gov identifier: NCT00069784) trial with insulin [16]. Reassuringly, prior intensive therapy was associated with long-term reductions in CHD during the observational follow-up studies of both the DCCT and UKPDS [17, 18], although the Action to Control Cardiovascular Risk in Diabetes (ACCORD; ClinicalTrials.gov identifier: NCT00000620) study group reported previously unrecognized harm from intensive glucose lowering in patients with type 2 diabetes with established cardiovascular disease or additional cardiovascular risk factors [19].

Dyslipidemia is a common feature of diabetes [20]. There is an association between atherosclerotic cardiovascular disease and serum cholesterol and triglyceride levels in both type 1 and type 2 diabetes [21, 22]. The risk of CHD is greater at any given level of serum cholesterol in patients with diabetes and its association with hypertriglyceridemia is stronger than in the general population [23]. Importantly, there is strong and convincing evidence that cholesterol lowering therapy significantly reduces CHD in patients both with and without diabetes [24–26]. There also appears to be no threshold below which a further reduction in low-density lipoprotein (LDL) cholesterol might be beneficial [25, 26].

Improved glycemic control generally has favorable effects on lipoprotein levels in diabetes, with a reduction in cholesterol and triglyceride levels through decreased circulating very-low-density lipoprotein (VLDL) and by increased catabolism of LDL through reduced glycation and upregulation of LDL receptors

[27, 28]. It is certainly possible that any cardiovascular benefit which might be derived from intensive glucose lowering is related to effects on lipoprotein metabolism rather than directly through altered glycemia [29].

This article is based on previously conducted studies and does not involve any new studies of human or animal subjects performed by any of the authors.

DYSLIPIDEMIA AND ATHEROSCLEROSIS IN DIABETES

The dyslipidemia of type 2 diabetes is characterized by high triglyceride levels and decreased high-density lipoprotein (HDL) cholesterol, changes observed many years before the onset of clinically relevant hyperglycemia [9, 30]. Recent evidence suggests that low HDL cholesterol is an independent factor not only for cardiovascular disease but also for the development of diabetes itself [31]. These changes, and the presence of small dense LDL particles, probably contribute to accelerated atherosclerosis even before diabetes is formally diagnosed [9, 32]. In type 1 diabetes, hypertriglyceridemia may occur, but HDL cholesterol levels are often normal or even high unless glycemic control is poor or nephropathy is present [30]. In addition, patients with diabetes show qualitative and kinetic abnormalities for all lipoproteins [33].

A number of factors may contribute to the alterations in lipid metabolism observed in patients with diabetes, including insulin deficiency or resistance, adipocytokines, and hyperglycemia [33]. Many aspects of the pathophysiology and consequences of diabetes dyslipidemia remain unclear, but the mechanism by which hypertriglyceridemia arises is fairly well understood [34]. Insulin deficiency or resistance activates intracellular hormone-sensitive lipase which increases the release of non-esterified fatty acids (NEFA) from triglycerides stored in the more metabolically active centrally distributed adipose tissue [35]. High circulating levels of NEFA increase hepatic triglyceride production. Increased hepatic triglyceride synthesis is associated with increased secretion of apolipoprotein B (apoB) [36] (see Fig. 1). Furthermore, the normal inhibitory effect of insulin on hepatic apoB production and triglyceride secretion in VLDL is lost, and the VLDL secreted is larger and more triglyceride-rich [37–39]. The tendency to hypertriglyceridemia is further augmented by reduced VLDL catabolism [33]. Lipoprotein lipase located on vascular endothelium largely determines the rate of removal of triglycerides from the circulation. In contrast to intracellular hormone-sensitive lipase this lipoprotein lipase may be downregulated in states of insulin resistance or deficiency [33]. This reduction in lipoprotein lipase activity also contributes to postprandial lipemia [40].

It is essential to rectify the commonly held misconception that triglyceride concentration is a poor indicator of cardiovascular risk. There is a strong relationship between triglycerides and CHD in both type 1 and type 2 diabetes. Raised serum triglycerides herald the development of type 2 diabetes mellitus, particularly when associated with other features of metabolic syndrome or CHD, and once diabetes has developed they continue to predict CHD risk, often independently of other risk factors [21]. Triglycerides are positively correlated with cholesterol, obesity, glucose intolerance, cigarette smoking, and hyperuricemia, and are negatively correlated with HDL cholesterol. When these factors are

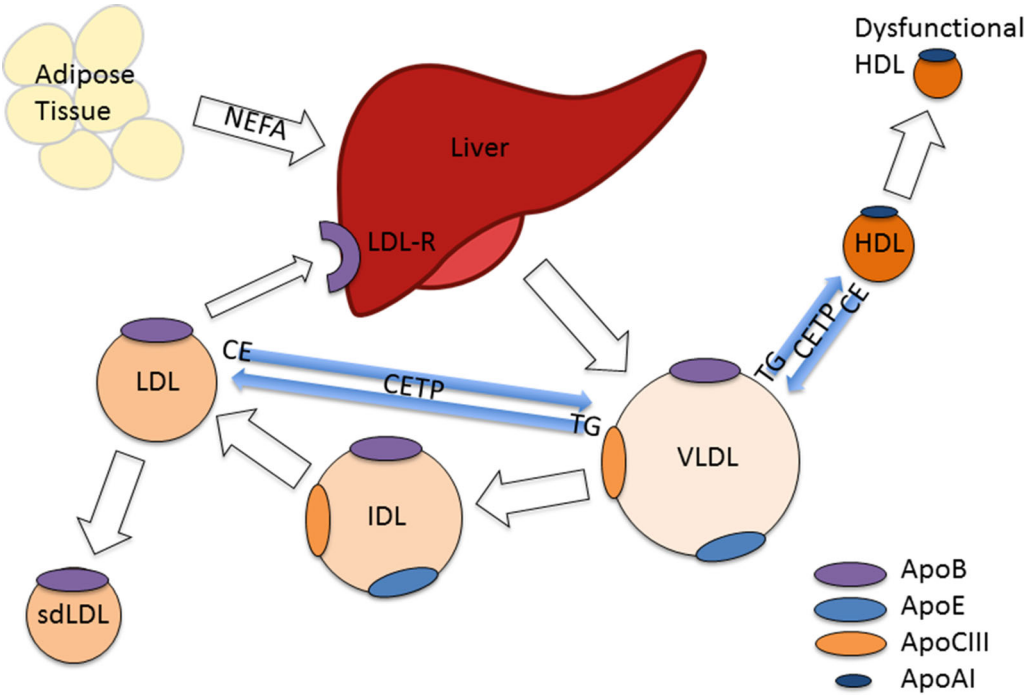

Fig. 1 Qualitative changes in lipoproteins in diabetes. *ApoAI* apolipoprotein AI, *ApoB* apolipoprotein B, *ApoCIII* apolipoprotein CIII, *ApoE* apolipoprotein E, *CE* cholesteryl esters, *CETP* cholesteryl ester transfer protein, *HDL* high-density lipoprotein, *IDL* intermediate-density lipoprotein, *LDL* low-density lipoprotein, *LDL-R* low-density lipoprotein receptor, *NEFA* non-esterified fatty acids, *sdLDL* small dense low-density lipoprotein, *TG* triglycerides, *VLDL* very-low-density lipoprotein

included in multivariate analysis, the element of risk attributable to triglycerides themselves appears less significant, but the risk associated with hypertriglyceridemia is still substantial with fasting levels of 2.6–4.5 mmol/L associated with a twofold excess of CHD risk and levels of 4.5–9.0 mmol/L with up to a ninefold elevation [41, 42].

Triglyceride-rich lipoproteins (chylomicrons and VLDL) are not known to participate directly in atherogenesis, but they are central to the mechanism by which small dense LDL cholesterol is generated and HDL cholesterol levels are depressed in diabetes [1]. Cholesteryl ester is transferred from other lipoproteins into the enlarged circulating pool of triglyceride-rich lipoproteins by cholesteryl ester transfer protein, and the rate of transfer is increased in both type 1 and type 2 diabetes [43, 44]. There is transfer of triglyceride in the opposite direction such that cholesteryl ester-depleted HDL and LDL become triglyceride-rich. The subsequent removal of this triglyceride by hepatic lipase results in smaller, denser HDL and LDL particles.

Levels of small dense LDL are thus increased, and the apparently relatively undisturbed cholesterol and apoB levels observed in many patients with diabetes may thus hide a major atherogenic change [45–47]. Circulating LDL is not believed to participate directly in atherogenesis, but must first undergo structural modification to allow its apoB to act as a ligand for the scavenger receptors of monocyte macrophages in the arterial wall, triggering foam cell formation [48]. The

proportion of glycated apoB is doubled even in reasonably well-controlled diabetes [28]. Glycated LDL may be more susceptible to oxidation or itself represent an atherogenic modification [49, 50]. Glycation of LDL also decreases its LDL receptor-mediated catabolism [51].

In patients with type 1 diabetes with good glycemic control, insulin upregulates lipoprotein lipase, increasing the production of small HDL particles [52, 53], frequently to higher than normal HDL cholesterol levels [54]. HDL cholesterol in diabetes has thus not arisen physiologically; HDL dysfunction is suggested by the observation that high HDL cholesterol levels do not always protect against CHD [55, 56]. HDL may be dysfunctional in its capacity to protect LDL against atherogenic modification. Reductions in the antioxidative and anti-inflammatory effects of HDL have been reported in patients with diabetes, alongside an impaired ability of HDL to counteract the inhibition of endothelium-dependent vasorelaxation by oxidized LDL [34, 57]. The role of HDL in protecting pancreatic beta cells against apoptosis is an important emerging area of research [58]. Glycation has recently been shown to reduce the sphingosine-1-phosphate content of HDL, reducing its ability to activate protective intracellular survival pathways during oxidative stress [59].

With the development of nephropathy, increased catabolism of smaller HDL particles likely combines with increased cholesteryl ester activity to lower HDL cholesterol levels [6, 60]. Glycation of HDL further enhances its catabolism [61]. Diabetes thus leads to impaired reverse cholesterol transport through both reduced HDL concentrations and HDL dysfunction [36]. Low HDL cholesterol appears to be of greater importance in patients at high cardiometabolic risk and is amongst the factors that currently favor a decision to further reduce LDL cholesterol levels [20].

CLINICAL INVESTIGATIONS

The apparently normal serum cholesterol concentrations observed in both type 1 and type 2 diabetes led to the widespread erroneous belief that glycemia alone might explain the observed high CHD rates. However, the relatively normal cholesterol levels hide an atherogenic lipid profile, with increased intermediate-density lipoprotein and small dense LDL, and dysfunctional HDL [49–51].

Each LDL particle, regardless of its density or cholesterol content, contains only a single tightly bound molecule of apoB. The often "normal" level of LDL cholesterol seen in many patients with diabetes actually disguises an increased particle number (higher apoB) and that clearance of small dense particles is slower [20]. Thus, measurement of the serum concentration of apoB provides a more discriminating index of atherogenic risk or therapeutic response than LDL cholesterol [62]. While intensive therapy in the DCCT did not significantly affect LDL and HDL cholesterol levels, it was associated with decreased apoB (and lipoprotein(a)) and with favorable alterations in lipoprotein subclasses that are not revealed by the standard lipid profile, but have implications for the evolution of both microvascular and macrovascular complications [63].

The use of LDL cholesterol in diabetes also underestimates the atherogenic contribution of triglyceride-rich particles so non-HDL cholesterol is a better measure of atherogenicity in diabetes [64]. The introduction of non-HDL cholesterol appears

to have obviated the need to introduce apoB measurements more widely in patient management.

DIET AND WEIGHT MANAGEMENT

Lifestyle modifications are the first-line intervention in the management of diabetes dyslipidemia, and include weight loss, dietary modification, and aerobic exercise [36]. Obesity increases insulin resistance and is associated with increased triglycerides and LDL cholesterol and decreased HDL cholesterol [1]. Weight loss is known to be associated with improvements in lipids and other cardiovascular risk factors including the incidence of type 2 diabetes [65, 66] and should therefore be encouraged in overweight patients with diabetes. To achieve sustained weight loss, caloric restriction remains the key and even modest degrees of weight loss are associated with an improvement in glycemic control, HbA1c, and lipid profile [67].

Increased physical activity may provide some small adjunct to the effect of dietary restriction, but is unlikely to be successful on its own. Reduced fat intake, particularly of saturated fat, should also be encouraged [68]. The American Diabetes Association (ADA) recommends a diet low in trans fat, saturated fat, and cholesterol [69]. In patients without a marked increase in serum triglycerides but who are not obese, some substitution of saturated fat can be made with unrefined carbohydrate foods and some with oleic, linoleic, or omega-3 fish oils [70].

Dietary interventions, while considered first-line treatment for all patients with diabetes, have not been successful in demonstrating a mortality benefit, even with prolonged follow-up [71].

The gastrointestinal lipase inhibitor orlistat causes fat malabsorption and should be taken close to meals. The patient must adhere to a low fat diet or they will experience steatorrhoea. There is often early benefit, but then weight loss levels out, likely as the patient learns to omit it if they plan to consume a fatty meal. Nonetheless any weight loss achieved can improve cardiovascular risk factors [72]. Orlistat has beneficial effects on serum total and LDL cholesterol levels which are greater than might be explained by weight loss alone [73].

Surgical management of obesity is much more effective than medical treatment [65]. Weight loss after bariatric surgery is also associated with beneficial glycemic effects in diabetes, including achievement of near normal glycemia without medication or reduced medications [74].

EFFECTS OF HYPOGLYCEMIC AGENTS ON LIPOPROTEINS

Diabetes dyslipidemia can be partly corrected by insulin treatment and improved blood glucose control [75]. Insulin therapy increases HDL cholesterol and reduces circulating triglyceride levels, particularly in patients with poor glycemic control [76]. Metformin decreases serum triglycerides and improves insulin resistance but is often overlooked as a lipid-lowering agent and is generally considered only as a hypoglycemic agent in the management of diabetes [77]. Other drugs used in the management of diabetes may also have unintended positive and negative effects on lipoproteins (see Table 1). Of particular interest is the small increase in LDL cholesterol observed following treatment with sodium-glucose cotransporter 2 (SGLT2)

Table 1 Effects of hypoglycemic agents on lipoproteins

Drug	Total cholesterol	LDL cholesterol	HDL cholesterol	Triglycerides	References
Metformin	↓↔	↓	↔↑	↓↔	[114]
Gliclazide	↓	↔	↔	↓	[115, 116]
Glimepiride	↔	↔	↔↑	↔	[115, 117]
Pioglitazone	↑	↔	↑	↓	[118, 119]
Sitagliptin	↔	↔	↔↑	↔	[120, 121]
Saxagliptin	↔	↔	↔	↔	[116, 122]
Vildagliptin	↔	↔	↔↑	↔	[123]
Linagliptin	↔	↔	↔	↔	[124]
Dapagliflozin	↔↑	↔↑	↔↑	↓↔	[125, 126]
Canagliflozin	↑	↑	↑	↑	[121, 127]
Empagliflozin	↔↑	↔↑	↔↑	↔	[78, 128]
Exenatide	↓↔	↔↑	↔↑	↓	[119, 129]
Liraglutide	↔	↔	↔	↓	[130, 131]

↑ Increased, ↔ no change, ↓ decreased, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

inhibitors, suggesting that the recently reported significant improvement in cardiovascular outcomes with empagliflozin is unrelated to effects on dyslipidemia [78].

LIPID-LOWERING DRUGS

The discovery of statins was a key advance in cardiovascular medicine. Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in cholesterol biosynthesis. There is a wealth of clinical trial evidence that lowering serum cholesterol with statins decreases the risk of CHD [24, 79]. These trials have demonstrated that statins decrease the risk of both CHD and stroke in people with and without pre-existing cardiovascular disease [24, 80]. It would appear from such trials that the relative risk reduction achieved with statin treatment is similar in patients with diabetes to that in other people. However, the number needed to treat to prevent one event (NNT) will be lower in patients with diabetes compared to those without diabetes but apparently similar lipid profiles [81].

The case for secondary prevention with statin therapy in diabetes is accepted, but whether all patients with diabetes should be considered for statin treatment has been more contentious. In the Heart Protection Study (Controlled-Trials.com identifier: ISRCTN48489393) the 2912 patients with diabetes without pre-existing vascular disease randomized to receive simvastatin showed a significant reduction in cardiovascular end-points compared to those allocated to placebo [82]. Similarly, in the Collaborative Atorvastatin Diabetes Study (CARDS; ClinicalTrials.gov identifier: NCT00327418), where the mean pretreatment LDL cholesterol was 3.0 mmol/L (<2.5 mmol/L in 25% of patients), the effect of active intervention with

atorvastatin was so favorable that the study was stopped early [24]. In both studies there did not appear to be any threshold below which statin therapy ceased to be beneficial. A meta-analysis of 18,686 people with diabetes from 14 randomized trials (1466 with type 1 and 17,220 with type 2) demonstrated a 9% reduction in all-cause mortality for every 1 mmol/L reduction in LDL cholesterol [26]. The effects of statin therapy were similar irrespective of baseline characteristics and prior history of vascular disease. Although the majority of participants in these trials had type 2 diabetes, the reduction in major vascular events was also statistically significant in people with type 1 diabetes.

The risk factor reduction brought about by statins occurs in a dose-dependent fashion, with higher dose statins associated with a greater lowering of cardiovascular events [83]. It should also be noted that in diabetes, in contrast to patients without diabetes, statins do not stop the progression of carotid intima media thickness or intravascular ultrasound-measured atheroma volume at typical doses, implying that high doses may be necessary to prevent atheroma progression [84]. One in seven patients with diabetes treated with statins still goes on to suffer a cardiovascular event over 5 years [26].

Clinical trial evidence therefore provides unequivocal evidence to support prescribing statins for both primary and secondary prevention in diabetes, but the populations studied may not be representative of younger patients or those with advanced renal disease. This requires further exploration and thus the exercise of clinical judgment in prescribing.

Interestingly, statin treatment is associated with a slight increase in the incidence of type 2 diabetes. A meta-analysis showed 4 years of statin treatment in 255 patients would lead to one extra case of type 2 diabetes [85]. However, this risk is low both in absolute terms and when compared with the expected cardiovascular benefit from reducing LDL cholesterol. In the same period 5.4 vascular events would be avoided in these 255 patients [85]. Clinical practice in patients with existing cardiovascular disease or moderate or high cardiovascular risk should not change.

Ezetimibe blocks the absorption of dietary cholesterol and the reabsorption of cholesterol entering the small intestine in bile, the latter accounting for most of its LDL cholesterol lowering. Ezetimibe is concentrated in the cells of the intestinal brush border, where it inhibits cholesterol absorption by a process which involves binding to Niemann-Pick C1-Like 1 [86]. It remains a second-line option for LDL cholesterol lowering in diabetes and has its greatest clinical utility as an adjunct to statin therapy.

The bile acid sequestrant colesevelam has been shown to reduce HbA1c in addition to reductions in total cholesterol, LDL, and non-HDL cholesterol levels [87]. Cholestyramine may be more effective in lowering LDL cholesterol, but both agents may increase triglycerides, and neither is particularly well tolerated [88].

Many novel therapies are in development to reduce LDL cholesterol, but none has been studied specifically in patients with diabetes [89]. In particular, proprotein convertase subtilisin/kexin 9 (PCSK9) inhibitors have emerged as medications showing significant reductions in LDL, with recent data suggesting similar effects on lipoproteins in patients with type 2 diabetes to those seen in patients without diabetes [90]. An additional potentially beneficial effect on postprandial

hypertriglyceridemia is suggested by novel data on PCSK9 regulation of intestinal lipoprotein assembly and secretion [91]. Ongoing cardiovascular outcome trials will inform the use of PCSK9 inhibitors in diabetes.

The ACCORD study showed an increased number of cardiovascular events in patients with diabetes and persistently low HDL cholesterol and high triglyceride levels, despite a mean LDL cholesterol below 2.1 mmol/L [92]. Fibrates act as peroxisome proliferator-activated receptor (PPAR)-α agonists to reduce triglycerides and modestly increase HDL cholesterol but also affect multiple pathways linked to the retinoid-X receptor [93]. To date no decrease in cardiovascular outcomes has been convincingly demonstrated in clinical trials [94]. In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD; Controlled-Trials.com identifier: ISRCTN64783481) study 9795 people with type 2 diabetes were randomized to receive micronized fenofibrate or placebo [95]. By the end of the trial statin treatment became a major confounding factor, but the primary end-point (fatal CHD and non-fatal myocardial infarction) decreased by 11% on fenofibrate compared to placebo. Although pretreatment serum triglyceride levels appeared to have no influence on the relative decrease in cardiovascular events, the overall effect was not significant, and the FIELD study did not establish a firm place for fibrate drugs in the management of diabetes dyslipidemia. More recently the ACCORD-LIPID trial reported no cardiovascular benefit from the addition of fenofibrate to simvastatin in patients with type 2 diabetes [92]. However, there did appear to be a beneficial effect on CHD outcomes in patients with triglycerides above 2.4 mmol/L and HDL cholesterol below 0.79 mmol/L [92]. There may therefore be a role for fibrates as adjunctive therapy in patients with diabetes and persistently elevated triglycerides. However, fibrates, particularly fenofibrate, may cause paradoxical reductions in HDL cholesterol levels and it is important that clinicians are aware of this phenomenon [96].

Most hypertriglyceridemia in diabetes is mild to moderate and statins remain the drug of first choice even in patients with mixed dyslipidemias, but in patients with genetic susceptibility severe hypertriglyceridemia may develop, with attendant risk of pancreatitis [97]. When triglycerides are above 11 mmol/L a fibrate should be considered first to reduce triglycerides and the risk of pancreatitis.

Purified omega-3 fatty acids can lower triglycerides as much as fibrates, but they have little effect on HDL or LDL cholesterol [98]. Omacor® (Abbott Healthcare, Abbott Park, IL, USA) contains 90% omega-3 fatty acid ethyl esters (mostly eicosopentaenoate or docosahexaenoate) and in combination with a statin can decrease triglycerides by a further 30%, most probably by inhibition of hepatic triglyceride synthesis [99]. Despite evidence that omega-3 fatty acids stabilize atheromatous plaques and reports of beneficial hypotensive and antithrombotic effects, a recent trial of omega-3 fatty acids in patients with metabolic syndrome or type 2 diabetes disappointingly found no effect on CHD risk [100, 101].

Another future therapeutic option may be the dual PPAR-α/γ agonist saroglitazar, which was approved in India in 2013 and has been shown to significantly reduce plasma triglyceride, total cholesterol, non-HDL cholesterol, and VLDL cholesterol, and HbA1c and fasting glucose levels [102].

NATIONAL AND INTERNATIONAL RECOMMENDATIONS FOR THE MANAGEMENT OF HYPERLIPIDEMIA

The role of diabetes in risk assessment processes is more controversial than the simple statement often applied in guidelines that diabetes is a cardiovascular disease-risk equivalent. Risk in type 1 diabetes is strongly related to glycemic control, nephropathy, and hypertension and can be significantly increased compared with normoglycemic subjects [103]. Risk in type 2 diabetes is still widely considered to be increased two- to fourfold [3, 104]. While features such as the presence of nephropathy or retinopathy identify higher risk groups, the use of other biomarkers of risk and likely need for enhanced treatment are often not appreciated. The presence of microalbuminuria for example is a risk factor for CHD even at low levels and its severity is also predictive of future events [105].

Most clinical guidelines recommend tight control of dyslipidemia, especially in high risk patients [106, 107]. Importantly, lipid targets are easier to achieve than blood pressure or glycemia targets and target LDL cholesterol levels as low as 1.8 mmol/L are increasingly recommended in patients with established CHD [108, 109].

A recent position statement from the ADA recommends a screening lipid profile at the time of diagnosis, at age 40 years, and periodically thereafter [110]. Treatment recommendations beyond lifestyle modification and optimization of glycemic control are for the use of high-intensity statin therapy (e.g., atorvastatin 40–80 mg or rosuvastatin 20–40 mg) in patients of all ages with overt CHD, and those aged 40–75 years with additional risk factors, and moderate intensity statin therapy (e.g., atorvastatin 10–20 mg or simvastatin 20–40 mg) for patients aged over 40 years without additional risk factors. Clinical judgment should guide the use of moderate or high-intensity statin therapy in patients younger than 40 years or older than 75 years with additional risk factors [110].

The most recent American Heart Association/American College of Cardiology guidelines for cholesterol management appear less interventional, recommending statin treatment only for patients with diabetes with clinical atherosclerotic cardiovascular disease or if aged 40–75 years [111]. There is also a modest recommendation for additional treatment in persons with statin intolerance or an inadequate response, with an emphasis on clinical judgment. The European Society of Cardiology suggests that ezetimibe be added after intensification of statin therapy in diabetes [112].

The National Institute for Health and Care Excellence also recently updated its guidance and now advises clinicians to offer statin treatment for primary prevention to adults with type 1 diabetes who are over 40 years, have had diabetes for more than 10 years, or have established nephropathy or other cardiovascular risk factors [113]. These guidelines also recommend statin therapy for primary prevention in type 2 diabetes if the 10-year risk of developing cardiovascular disease is estimated to be greater than 10% using the QRISK2 assessment tool [113].

In conclusion, statin treatment should effectively be considered for all people with diabetes aged over 40 years or younger if additional cardiovascular risk factors are present, a position recently supported by the Joint British Societies [106].

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Diabetic dyslipidaemia.

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Diabetic dyslipidaemia

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Purpose of review
The purpose is to discuss recent developments in the understanding of lipoprotein metabolism in diabetes, the cardiovascular risk associated with both type 1 and type 2 diabetes, recently published guidelines on the management of this risk, concerns over the use of statin treatment in diabetes, and other therapeutic options.

Recent findings
Diabetic dyslipidaemia can be gross with massive hypertriglyceridemia, or subtle with a lipid profile which would be regarded as normal in a nondiabetic patient, but which hides underlying increases in atherogenic subfractions of LDL (e.g., small dense LDL, glycated LDL) and remnant lipoproteins. Statins can decrease these without the clinician being aware from routine biochemistry. In type 2 diabetes, HDL cholesterol levels are often reduced, whereas in type 1, insulin can raise HDL, but its antiatherogenic properties are compromised. Dyslipidaemia and hypertension predate the onset of glycaemia of diabetic proportions (metabolic syndrome). Obese people can thus die of diabetes before they develop it. Obesity should be prevented and treated. Statins decrease the risk of cardiovascular disease in diabetes or metabolic syndrome regardless of whether glycaemia worsens.

Summary
One unassailable truth is that statin therapy is beneficial and should rarely, if ever, be withheld.

Keywords
diabetes dyslipidaemia, glycated LDL, hypertriglyceridemia, lipid modifying drugs, obesity

INTRODUCTION

Diabetes represents a major disturbance of all three systems for the transport of lipid energy, namely fatty acids, ketone bodies, and lipoproteins. Ketoacidosis, atherosclerotic cardiovascular disease (CVD) and, in part, microvascular complications are the consequence. This review will focus on recent publications on lipoproteins in diabetes obtained through PubMed: earlier references can be found in a previous review [1].

Evidence that statin treatment can substantially decrease the risk of atherosclerotic CVD in type 2 diabetes mellitus (T2DM) [2] was assimilated into guidelines for diabetes management soon after its publication [3,4]. The same recommendations also embraced statin therapy in T1DM, although the clinical reasoning for this was different from that for T2DM [5]. The guidance was not enthusiastically received by all diabetologists, who continued to believe that all the problems associated with diabetes could be solved by regulating blood glucose. Many were prepared to prescribe new oral hypoglycaemic agents with abandon, despite no evidence of benefit or lack of harm [6▪], but frequently considered the CVD risk in diabetes to be insufficiently high enough for statin treatment, which they viewed with suspicion. This review will focus on recent advances in our understanding of CVD risk in diabetes before considering what is known about atherogenic lipoprotein metabolism in diabetes and metabolic syndrome and how diabetic dyslipoproteinemia interacts with statin therapy. The interpretation of lipid results from the clinical laboratory both before and after commencing statin treatment will be considered in the light of recent controversial recommendations to abandon therapeutic goals. New evidence about statin safety and the place of nonstatin lipid-lowering

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KEY POINTS

- Cardiovascular risk continues to be underestimated in diabetes, particularly in young patients.
- Statin treatment can substantially decrease the risk of atherosclerotic CVD in diabetes.
- Routine laboratory measurements do not reflect the atherogenic changes in lipoproteins in diabetes
- Medications used to manage hyperglycaemia can also affect the lipid profile unfavourably.

treatment in diabetes and the effect on lipids of hypoglycaemic agents will then be reviewed.

CARDIOVASCULAR RISK IN TYPE 1 AND 2 DIABETES

Recent recommendations for statin treatment in T2DM are in the USA to give atorvastatin 20 mg daily as first-line treatment when estimated CVD risk exceeds 7.5% in the next 10 years [7] and in the UK when CVD risk reaches 10% or more over the next 10 years [8]. Three important caveats must be considered with regard to risk estimation in diabetes. Firstly, the many rival risk engines which have been developed are all based on survivor populations and inevitably omit people who have already sustained CVD events before the observed cohort was assembled. As such, they will underestimate risk. The Renfrew and Paisley Study [9] is important because it observed people from a random population sample, who developed diabetes during a long period of follow-up. It revealed that in the first 5 years after the onset of T2DM CVD risk increases such that in the subsequent 5 years it reaches that of nondiabetic people with established CVD, and in the 5 years after that rises further. Secondly, very few, if any typical T2DM patients will not have achieved at least a 7.5 or 10% 10-year CVD risk by middle age. This means that it will be younger people who, as the result of risk assessment, will be denied statin treatment, yet the risk estimation is at its most inaccurate in younger people with diabetes, because they were not well represented in the populations from which data to design the risk engines were derived. We know that in people who are so insulin-resistant as to develop T2DM in youth either because of extreme obesity or a genetic cause, the lifetime risk of CVD is astronomic [10]. So any attempt to withhold treatment from them seems perverse. Thirdly, it is often seen as an advantage that the multivariate equations on which these risk engines are based have as variables, not only the mutable risk factors [lipids, blood pressure (BP), and smoking], but also age, sex, obesity indices, family history, HbA1c, and so on. Additional risk factors might modestly improve the proportion of observed CVD risk explained by the model, but each additional risk factor will erode the contribution of risk factors already in the equation. In biological terms this is because many risk factors such as obesity, age, and family history operate through cholesterol or BP; and in mathematical terms because the multiple correlation coefficient can never exceed unity so each additional risk factor added to the model will reduce the contribution of others. This leads to an apparent underestimation of the contribution, say of LDL cholesterol (LDL-C), to risk. We know from meta-analysis of randomized clinical trials which shows a 22% decrease in CVD risk for each 1 mmol/l that decreasing LDL-C has a substantially greater effect than predicted in currently recommended multivariate risk prediction models [11]. Recent work suggests that much of the excess CVD incidence in diabetes is because of inadequate treatment of lipids and BP [12].

Evidence that statins decrease CVD incidence in T1DM is derived from meta-analysis of T1DM participants included in trials not specifically aimed at recruiting them. In common with other higher risk subgroups their CVD risk decreased by about one-fifth for each 1 mmol/l reduction in LDL-C [5,11]. Concern has been expressed that these T1DM participants may not have been typical. However, although there is no satisfactory method of predicting CVD risk in T1DM, it is important not to be lulled into a false sense of security in lean, young adults with apparently normal lipid levels. T1DM often begins in childhood and a 20 year old may thus have had it for 15 or more years. Duration of diabetes is important and is easy to date in type 1, unlike type 2. Furthermore, even minimal evidence of nephropathy greatly increases CVD risk in T1DM [1]. In a large series of patients with T1DM 10-year CVD mortality exceeded 5% between the ages of 30 and 40 and this is likely to represent a combined 10-year CVD morbidity and mortality of 15–20% [13–15]. This conclusion is compatible with other studies reviewed in the recent scientific statement from the American Heart and Diabetes Associations [16]. So commencing statins in patients in their 20s is sensible, particularly if T1DM has been present for 10 years, or earlier if nephropathy has reached the stage of microalbuminuria, or hypertension has developed [17].

In both T1DM and T2DM, there must generally be a good reason to withhold statin treatment rather

than the reverse. One of the most disconcerting aspects of diabetes care to emerge recently is that the incidence of CVD deaths among young women with T1DM now exceeds that of men [18▪]. These young women are precisely the group in whom statins are most often withheld [19].

NATURE OF DYSLIPOPROTEINEMIA IN DIABETES AND METABOLIC SYNDROME

In both T1DM and T2DM, there is a tendency to hypertriglyceridemia [1]. This is typically more marked in T2DM where it results from hepatic overproduction of a large, triglyceride-rich VLDL (VLDL1). This competes with chylomicrons for clearance from the circulation, which is already compromised, because lipoprotein lipase activity in adipose tissue and muscle is decreased by peripheral insulin resistance. Simultaneously, hepatic insulin resistance removes the suppression of triglyceride synthesis and VLDL secretion normally exerted by insulin [20▪▪]. The suppression of triglyceride synthesis is overcome by the high flux of nonesterified fatty acids to the liver, released from the huge adipose tissue organ typically present, particularly viscerally. Insulin resistance promotes this, because the action of insulin on adipose tissue is to inhibit the intracellular lipase responsible for releasing fatty acids and glycerol from intracellular depots (the exact opposite of its action on the extracellular lipoprotein lipase responsible for clearance of triglyceride-rich lipoproteins) [1]. In the liver the excess triglyceride synthesis may overcome the diminished capacity for VLDL secretion, resulting in nonalcoholic steatohepatitis. The VLDL particles secreted are large and overloaded with triglyceride and are termed VLDL1. The expanded circulating pool of VLDL1 attracts cholesteryl ester from LDL and HDL in exchange for triglyceride under the agency of cholesteryl ester transfer protein (CETP), the activity of which is thus increased [1]. As a consequence small cholesterol-depleted HDL and LDL particles are created. HDL cholesterol (HDL-C) is thus decreased, an effect further compounded by diminished lipoprotein lipase activity. The cholesterol-depleted LDL particles are stripped of the triglyceride they have received by hepatic lipase to become small dense LDL (sd-LDL). Because they do not contribute greatly to LDL-C their presence cannot be detected by routine laboratory results provided to the clinician. That they continue to contribute to circulating LDL-C could easily be shown were apoB (the major component of the protein moiety of LDL and VLDL) to be measured [21]. The insistence by some authorities that non-HDL-C can substitute for apoB is largely because of studies of nondiabetic populations in which dyslipoproteinemia was not prevalent [22]. The clinician must infer the presence of sd-LDL from the low HDL. Low HDL is often the most marked abnormality in the lipid profile of T2DM and often predates the onset of metabolic syndrome [23▪▪]. Triglycerides can be markedly elevated in diabetes, but are frequently more modestly increased [1]. Levels of triglycerides, however, need only exceed 1.5 mmol/l for sd-LDL to be generated [24]. Even when LDL-C is less than 2.5 mmol/l (100 mg/dl) CVD risk is increased when the effects of low HDL are compounded by moderately raised triglycerides [25▪▪]. The view based on studies of the general population that triglycerides are of little consequence in predicting CVD risk is misleading in understanding diabetic dyslipidaemia [1]. The presence of large VLDL1 can be detected in the circulation before the development of T2DM [26▪▪]. The regulation of VLDL metabolism was recently extensively reviewed [27].

In metabolic syndrome a dyslipidaemia similar to T2DM occurs well before blood glucose rises to diabetic proportions. The dyslipidaemia tends to worsen with time, but it can be severe enough to present as combined hyperlipidaemia before the onset of diabetes or, untreated, to cause CVD events, explaining why many patients become diabetic (on glucose criteria) after a CVD event: they were already well on their way to diabetes [28]. Some, in whom the presenting cardiovascular event proves fatal, will therefore actually die of diabetic dyslipidaemia before their glucose has reached diabetic proportions. It is precisely this type of patient with metabolic syndrome likely to progress fairly soon to T2DM, in whom a small rise in fasting glucose after commencing statin treatment means they cross the threshold for diagnosis of diabetes (see later).

The particular importance of the presence of sd-LDL frequently hidden in diabetes and metabolic syndrome is that it is highly atherogenic. Unlike more buoyant LDL, it is not cleared by the physiological LDL receptor. Its circulating half-life is thus extended, perhaps additionally too because it tends to be retained in tissues, including the subintimal space of the arterial wall. It is more susceptible to chemical modifications such as oxidation (oxLDL) and glycation (glycLDL) [29,30]. Indeed, the circulating level of glycated apoB in both diabetic and nondiabetic people is strongly determined by the concentration of sd-LDL (Fig. 1) [29]. Compared with buoyant LDL, both oxLDL and glycLDL are markedly more rapidly taken up by macrophage scavenger receptors to form foam cells and this is believed to be the basis of atherosclerosis. Statin

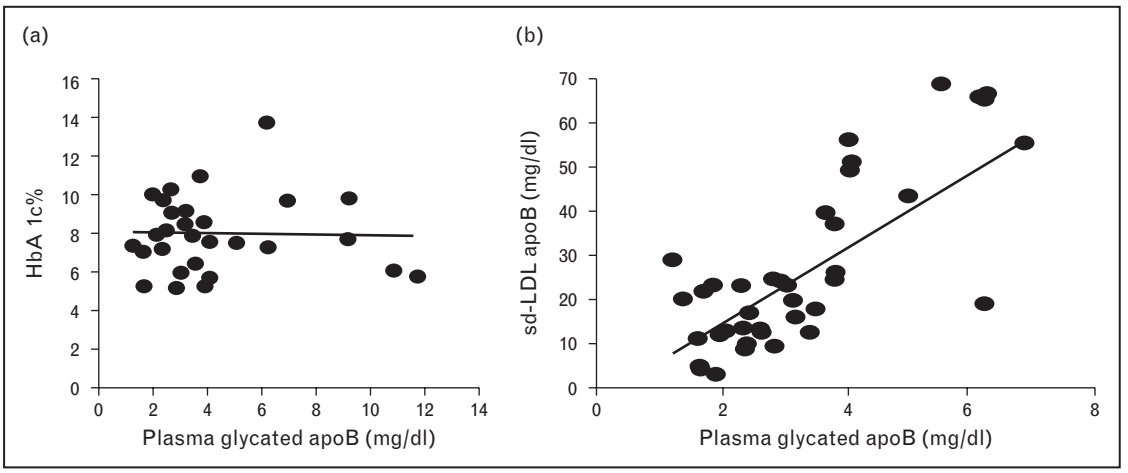

FIGURE 1. Plasma concentration of glycated apoB, HbA1c and sd-LDL apoB. The plasma concentration of glycated apoB in type 2 diabetes plotted as a function of serum HbA1c (a) ($r = 0.01$; p NS) and sd-LDL-apoB (b) ($r = 0.74$; $P < 0.01$). Glycated apoB, glycated apolipoprotein B; HbA1c, glycated haemoglobin; sd-LDL apoB, small dense low-density lipoprotein apolipoprotein B100.

treatment is probably effective because, in addition to lowering intermediate density lipoprotein (IDL) and LDL-C, it decreases circulating levels of sd-LDL, oxLDL, and glycLDL [29].

Lipoprotein metabolism in T1DM remains relatively poorly researched. In T1DM, although triglycerides can be grossly elevated, when glycaemic control is good they are frequently only modestly raised, even when the upper limit of normal is considered to be 1.5 mmol/l. Rapid triglyceride lipolysis will, however, mask an underlying increase in hepatic VLDL secretion (see later). LDL-C can be low and when glycaemic control is satisfactory HDL-C may be higher than in nondiabetic people [31–33]. Something nasty is, however, lurking in the woodshed. As in T2DM there is hepatic overproduction of VLDL not because of insulin resistance (unless obesity is also present, which it increasingly is in T1DM as it is in society generally and because many patients are fearful of hypoglycaemia), but because, in T1DM, systemic (subcutaneous) administration of insulin treatment means that in most patients there will be an excess of insulin activity in the tissues receiving blood systemically to provide a high-enough concentration in the liver via the hepatic artery to regulate liver glucose metabolism. Physiologically, a higher insulin level in the liver is achieved, because the concentration arriving via the portal vein is three to 10 times that in the systemic circulation. Almost certainly a deficiency of insulin action in the liver is what leads to the tendency to hypertriglyceridemia in T1DM. Ironically high-peripheral insulin exposure leads to enhanced clearance of triglyceride from the circulation because of upregulation of lipoprotein lipase to supraphysiological levels. This is probably the main reason that higher blood triglyceride levels are often not found and why in patients well controlled on insulin HDL-C levels are high, because hydrolysis of triglycerides by lipoprotein lipase releases components of HDL [1]. LDL-C levels are often not raised and may even be lower than in age-matched nondiabetic controls [31,32]. Even when obesity leads to superimposed metabolic syndrome in T1DM the lipid levels can appear to depart little from normal [34,35,36▪]. sd-LDL may, however, be produced as a consequence of the increased CETP activity which has been reported in T1DM as well as T2DM [37,38], but this requires confirmation. The consequence of the rapid hydrolysis of triglycerides may also lead to a build-up of IDL and chylomicron remnants, particularly if glycation of apoE [39] impairs their hepatic clearance from the circulation. Attention was drawn to the increase in the cholesterol to apoB ratio in T1DM, a phenomenon seen in familial dysbetalipoproteinemia, which like diabetes is associated with peripheral arterial disease as well as coronary heart disease [31]. Whether apoE, ox-LDL, and glyc-LDL levels are increased in T1DM patients has never been reliably reported. Their high risk of CVD, which is ameliorated by statins, thus remains poorly understood. Their high-circulating HDL is also no salvation as it is probably dysfunctional [40], lacking components such as paraoxonase 1 important for its antioxidant and antiglycative roles [41▪,42,43].

In T1DM, as in T2DM, the occurrence of nephropathy can dramatically increase cholesterol and triglyceride concentrations whereas HDL-C declines often to the low levels more typical of high vascular

disease risk patients [1]. Lipoprotein(a) also increases in nephropathy [32,44].

NONPHARMACOLOGICAL THERAPIES

Diet

The avoidance of obesity (the cause of most insulin resistance) through diet and, if practical, exercise is clearly important in both T1DM and T2DM. Simple sugar should also be avoided, but it is more important to restrict saturated fat than unrefined carbohydrate. Carbohydrate intake will be matched to insulin treatment in T1DM and it is important that the insulin regimen chosen does not lead to weight gain through fear of hypoglycaemia.

Bariatric surgery

Bariatric surgery-induced weight loss is associated with a significant improvement in lipid profile in obese patients with and without diabetes [45,46,47▪▪]. We recently reported favourable changes in HDL functionality and composition, systemic inflammation, and perivascular adipose properties and anticontractile function [45,48,49].

PHARMACOTHERAPY

Statins

Statin treatment should not be withheld without good reason [2]. There is stronger evidence for statins than for any other oral medication, including hypoglycaemic agents and antihypertensive patients [5,11]. There is no suggestion that these other medications should not be given, but consideration of a statin should not be relegated for consideration until only after their initiation. The effectiveness of statins in diabetes is probably because they upregulate hepatic LDL (apoB/E) receptor clearance of chylomicron remnants, IDL, and buoyant LDL. The effect is both to diminish CETP activity necessary for formation of sd-LDL and to decrease its precursors [50,51]. The decrease in sd-LDL which is susceptible to both oxidative and glycative modification reduces the levels of the highly atherogenic ox-LDL and glyc-LDL [52]. Conventional clinical laboratory measurements of LDL or non-HDL-C are frequently a poor guide as to whether to treat or what the goal of treatment should be. In primary prevention, treatment should be initiated with atorvastatin 20 mg daily and in secondary prevention atorvastatin 80 mg daily [7,8,46]. If this does not decrease non-HDL-C to less than 2.5 mmol/l (or LDL to less than 1.8 mmol/l), the statin dose should be titrated up in the case of primary prevention and adjunctive lipid-lowering medication considered in secondary prevention [53]. This departs from advice to abandon lipid targets for statin therapy [7,8,46]; whereas we agree that this approach is reasonable for patients whose pretreatment of non-HDL-C or LDL-C is low enough for them to achieve values less than 2.5 or 1.8 mmol/l respectively on atorvastatin in the recommended doses (or another statin in equally effective doses), we do not consider it rational not to aim for these targets in people who fail to achieve them (generally because they have higher than average pretreatment levels) and to persist with the statin at its initial dose [54,55]. Statins are generally well tolerated and effective in diabetic nephropathy [56▪]. Much publicity has recently attended reports that statins cause T2DM and that they may cause deterioration in glycaemic control in established T2DM [57,58]. The placebo groups in statin trials, however, clearly reveal that the great majority of people developing diabetes would have done so anyway, as the metabolic abnormalities already present progress to glycaemia of diabetic proportions, and that the rate at which hypoglycaemic medication requires escalation is almost identical to that in statin-treated participants [59,60▪▪]. Most importantly the reduction in CVD risk of 38% in the Collaborative Atorvastatin Diabetes Study was achieved regardless of change in glycaemic control and in people developing diabetes on statins' CVD risk was reduced to the same extent as those who did not develop diabetes and was significantly lower than in people not randomized to receive statin treatment [60▪▪]. It is also interesting that the incidence of diabetes is unaffected by statin treatment in familial hypercholesterolemia [61].

Nonstatin lipid modifying drugs

Ezetimibe inhibits cholesterol absorption by a process which involves binding to Niemann–Pick C1-Like 1 protein [62]. It has its greatest clinical utility as an adjunct to statin therapy. The IMProved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) demonstrated additional lowering of LDL-C and incremental clinical benefit when adding ezetimibe to statin therapy [63].

The bile acid sequestrant colesevelam hydrochloride has been shown to reduce total cholesterol, LDL-C, apoB, and non-HDL-C levels [64]. Unlike earlier bile acid sequestrants, it also has favourable effects on glucose homeostasis and lowers HbA1c [65]. In 2008 the US Food and Drug Administration approved the use of colesevelam as an adjunct to

antidiabetes therapy for improving glycaemic control in adults with T2DM.

Fibrates act as peroxisome proliferator-activated receptor-α agonists to reduce triglycerides and modestly increase HDL-C, but also affect multiple pathways linked to the retinoid-X receptor [66]. In the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study 9795 people with T2DM were randomized to receive micronized fenofibrate or placebo [67]. The primary endpoint [fatal coronary heart disease (CHD) and nonfatal myocardial infarction] decreased by 11% on fenofibrate compared with placebo, but this was not statistically significant. The FIELD trial is difficult to interpret, because add-in statin treatment became a major confounding factor. Nevertheless, a significant reduction in CVD events was evident in the subgroup of participants with low levels of HDL-C and hypertension [68,69], and the largest reduction in CVD incidence was observed in fenofibrate-treated patients with marked dyslipidaemia [70]. The lipid component of the Action to Control Cardiovascular Risk in Diabetes (ACCORD-Lipid) trial reported no cardiovascular benefit from the addition of fenofibrate to simvastatin in patients with T2DM in the trial as a whole [71]. However, a beneficial effect on CHD outcomes was observed in patients with triglycerides more than 2.4 mmol/l and HDL-C less than 0.79 mmol/l [71]. There may therefore be a role for fibrates as adjunctive therapy in patients with diabetes and persistently elevated triglycerides and/or low HDL-C. As in the FIELD study, the progression of retinopathy was slowed by fenofibrate and both the rate of progression and severity of diabetic retinopathy were reported as determinants of CVD outcomes in a subsequent study [72]. Overall, there is evidence to support adding fenofibrate to ongoing statin therapy when CVD risk is particularly high when the diabetes is associated with high triglycerides and low HDL-C. [67–71]. This requires close clinical supervision, because of the increased risk of myopathy. The use of fibrates as monotherapy should, however, be discouraged, because, unlike statins, evidence that they decrease all-cause mortality is lacking [8].

Purified ω-3 fatty acids can lower triglycerides as much as fibrates, but they have little effect on HDL-C or LDL-C [73]. Omacor contains 90% ω-3 fatty acid ethyl esters (mostly eicosopentaenoate or docosahexaenoate) and in combination with a statin can decrease triglycerides by a further 30%, most probably by inhibition of hepatic triglyceride synthesis [74]. However, a trial of ω-3 fatty acids in patients with metabolic syndrome or T2DM disappointingly found no effect on CHD risk [75,76].

Effects of lipid modifying drugs are summarized in Table 1.

New lipid modifying therapies

Proprotein convertase subtilisin kexin 9 inhibitors have emerged as medications showing significant reductions in total cholesterol, LDL-C, non-HDL-C, triglyceride, and Lp(a) [77]. In two separate studies, in a post hoc and an exploratory analysis compared with placebo, LDL-C lowering with alirocumab and evolocumab therapy was associated with a significant reduction in the rate of cardiovascular events in patients receiving treatment with statins at the maximum tolerated dose [78,79]. Both evolocumab and alirocumab show promising results in T2DM with no evidence of any effects on glucose homeostasis [80,81].

Another future therapeutic option may be the dual proliferator-activated receptor-α/γ agonist Saroglitazar, which was approved in India in 2013 and has been shown to significantly reduce plasma triglyceride, total cholesterol, non-HDL-C, VLDL cholesterol, HbA1c, and fasting glucose levels [82].

8-Hydroxy-2,2,14,14-tetramethylpentadecanedioic acid (ETC-1002) is a small molecule shown to modulate pathways of cholesterol, fatty acid, and carbohydrate metabolism. In a single-centre, double-blind, placebo-controlled trial in 60 patients with T2DM, ETC-1002 monotherapy reduced total cholesterol, non-HDL-C, and LDL-C significantly compared with placebo but with no significant effect on triglyceride concentration or glycaemia [83].

GLUCOSE-LOWERING AGENTS

Medications used to control glucose in patients with diabetes have various effects on the lipid profile [84], these effects are summarized in Table 2.

SEVERE HYPERTRIGLYCERIDEMIA

Hypertriglyceridemia in diabetes is usually mild-to-moderate and statins remain the drug of first choice even in patients with mixed dyslipidaemias and low HDL-C. A small proportion of patients with genetic susceptibility (generally heterozygous lipoprotein lipase deficiency) may develop severe hypertriglyceridemia with a tendency to develop chylomicronemia, associated with an increased risk of acute pancreatitis [85]. Instruction in a very low-fat diet (<20 g/day) to stem the production of chylomicrons should be provided by a specialist dietician. There is no good evidence that lipid-lowering drugs can improve matters, although fibrates are generally

Table 1. Effects of lipid modifying agents on lipoproteins and glucose metabolism

Drug	TC	LDL-C	HDL-C	TG	apoB	Non-HDL-C	Lp(a)	Effect on glycaemia[a]
Statins	↓	↓	↔↑	↓↔	↓	↓	↔	↑
Ezetimibe	↓	↓	↔	↔	↓	↓	↔	↔
Fibrates	↓	↓	↓	↓	↓	↓	↔	↔↓
Colesevelam	↓	↓	↔	↔↑	↓	↓	↔	↓
PCSK9 monoclonal antibodies	↓	↓	↔	↓↔	↓	↓	↓	↔
CETP inhibitors[b]	↓↔	↓↔	↑	↓↔	↓↔	↓	↓[c]	↓[b]
Purified omega-3 fatty acids	↔	↔	↔↑	↓	↔	↔	↔	↔
ETC-1002	↓	↓	↔	↔	–	↓	–	↔

↓, decrease; ↑, increase; ↔, no change; –, no data on some medications in the class; ApoB, apolipoprotein B100; CETP, cholesteryl ester transfer protein; ETC-1002, 8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid; LDL-C, LDL cholesterol; non-HDL-C; non-HDL cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; TC, total cholesterol; TG, triglycerides.
[a]Limited data available for other classes compared with statins.
[b]CETP inhibitor anacetrapib is reported to improve insulin sensitivity and reduceHbA1c.
[c]Anacetrapib and TA-8995 reduce Lp(a).

prescribed. Bile acid sequestrants should be discontinued as they exacerbate hypertriglyceridemia. A statin is generally given, not because under these circumstances it will discernibly decrease triglycerides, but because it is likely to increase removal of chylomicron remnants and IDL from the circulation and thus decrease CVD risk. They are also highly effective in patients who combine familial dysbetalipoproteinemia with diabetes. The combination of statin and fibrate increases the potential for myositis, but with patient awareness and regular monitoring the risk is manageable. Metformin and insulin also have a triglyceride-lowering effect. If ω-3 fatty acids are given, there should be a highly purified preparation to avoid increasing fat ingestion. Also as they are highly susceptible to oxidation, the patient should be carefully evaluated to ensure that they have not contributed to the frequency of attacks of

Table 2. Effects of hypoglycaemic agents on lipoproteins

Drug	TC	LDL-C	HDL-C	TG
Metformin	↓↔	↓	↔↑	↓↔
Gliclazide	↓	↔	↔	↓
Glimepiride	↔	↔	↔↑	↔
TZDs				
Pioglitazone	↑↓	↑	↑	↓
PPAR α/γ agonist				
Saroglitazar	↓	↓[a]	↑	↓
DPP-4 inhibitors				
Sitagliptin	↔	↔	↔↑	↔
Saxagliptin	↔	↔	↔	↔
Vildagliptin	↔	↔	↔↑	↔
Linagliptin	↔	↔	↔	↔
GLP1 analogues				
Exenatide	↓↔	↔↑	↔↑	↓
Liraglutide	↔	↔	↔	↓
SGLT2 inhibitors				
Dapagliflozin	↔↑	↔↑	↔↑	↓↔
Canagliflozin	↑	↑	↑	↑
Empagliflozin	↔↑	↔↑	↔↑	↔
Insulin therapy	↔↓	↓	↔↑	↓

DPP4, dipeptidyl peptidase-4; GLP1, glucagon-like peptide-1; SGLT2; sodium/glucose cotransporter 2; TZDs, thiazolidinedione.
[a]Only higher dose of saroglitazar 4 mg daily reduced LDL-C and apolipoprotein B but no significant change in the group treated with saroglitazar 2 mg daily. This product is licensed in India since 2013.

acute pancreatitis. There is anecdotal evidence that high-dose antioxidants can decrease episodes of pancreatitis even though they have no effect on lipid levels [86].

Apolipoprotein C-III is a key regulator of plasma triglyceride levels. A second-generation antisense inhibitor of apolipoprotein C-III synthesis was reported to reduce triglycerides significantly among patients with hypertriglyceridemia, including familial chylomicronemia syndrome, by up to 86% [87,88].

Alipogene tiparvovec (Glybera) is an adeno-associated virus serotype 1-based gene therapy for adult patients with familial lipoprotein lipase deficiency with a history of severe or multiple pancreatitis attacks despite dietary fat restrictions [89]. It is administered by multiple intramuscular injections. Triglyceride levels decreased initially but returned to pretreatment levels within 16–26 weeks after administration. However, sustained improvements in postprandial chylomicron metabolism were observed, and patients continued to express functional copies of the *LPL (S477X) gene* and of biologically active LPL in skeletal muscle [90]. Of more clinical relevance, after up to 6 years' follow-up after administration, there were clinically relevant reductions in the incidence of documented pancreatitis and acute abdominal pain events consistent with pancreatitis when compared with pretreatment history. Alipogene tiparvovec was generally well tolerated, with most adverse events being localized, transient, mild-to-moderate injection-site reactions [90–92].

CONCLUSION

CVD risk continues to be underestimated in diabetes through the use of risk engines based on survivor populations, which are not applicable to younger people, but which also include immutable risk factors. As a clinician it is important not to be lulled into a false sense of security when faced with a young patient with diabetes with an apparently unremarkable lipid profile. The use of LDL-C and non-HDL-C rather than apoB measurements fails to detect the presence of atherogenic sd-LDL in diabetes. Statin treatment should not be withheld in patients with diabetes without good reason.

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4. Glycation as an Atherogenic Modification of Lipoproteins, and the Contribution of Copper

Abstract

Background & Aims

Cardiovascular disease is the leading cause of death and disability in diabetes. Low-density lipoprotein (LDL) is the permissive factor in the development of atherosclerosis but must undergo oxidation and / or glycation to participate in atherogenesis. Glycated LDL is present in the circulation under physiological conditions and at higher concentrations in diabetes and in those destined to experience myocardial infarction, but LDL glycation *in vitro* requires supraphysiological glucose concentrations. Protection against glycation is a recognised function of high-density lipoprotein (HDL), which might itself be impaired by glycation. The role of copper in promoting *in vitro* glycation requires further investigation as it may be possible to manipulate this process therapeutically with a copper chelating agent to protect lipoproteins against glycation.

Materials & Methods

LDL (density 1.019 – 1.063 g/ml) and HDL (density 1.063 – 1.21 g/ml) were isolated from human serum using sequential preparative ultracentrifugation before incubation of LDL at a concentration of 1 mg/ml with glucose and copper at physiological concentrations (5 – 25 mmol/l and 1.25 – 5 mmol/l respectively) for 3 days. The ability of HDL and the divalent copper-selective chelator triethylenetetramine (TETA) to protect against glycation were also assessed. Glycated lipoproteins were separated from non-glycated lipoproteins using *m*-aminophenylboronate affinity chromatography and Apolipoprotein B (apo B) measured by a validated in-house high-sensitivity ELISA. Lipid hydroperoxide (LPO) formed during glycation was measured by a cholesterol oxidase colorimetric assay. The cholesterol efflux capacity of HDL *in vitro* was determined by a previously validated assay.

Results

In vitro glycation occurs more readily in the presence of physiological concentrations of copper. Incubation with 10 mmol/l glucose and 1.25 μmol/l and 2.5 μmol/l copper sulphate for 3 days significantly increased the proportion of glycated apo B ($P = 0.003$ and 0.01 respectively). Similar effects were observed in the presence of copper chloride ($P = 0.05$) but not copper-histidine complex or ferrous salts. HDL and copper-selective chelation with TETA both prevented glycation of apo B ($P = 0.02$). Glycation also appears to impair Paraoxonase-1 activity and the capacity of HDL to promote cholesterol efflux, an effect magnified by the addition of copper sulphate.

Discussion

This work offers insight into HDL functionality in diabetes and supporting evidence for the emerging link between copper dysregulation and the accumulation of advanced glycation end-products, and a potential role for copper in lipoprotein glycation.

Background and Aims

Cardiovascular Disease in Diabetes

Cardiovascular disease (CVD) due to atherosclerosis of the arterial wall and subsequent thrombosis is the leading cause of morbidity and premature mortality in Europe, and is increasingly common in developing countries [290]. In a meta-analysis of almost a million patients, diabetes mellitus was associated with a two- to threefold increased risk of death from CVD [163]. Although the relationship between diabetes and CVD is well established, the underlying processes remain poorly understood [291].

Atherosclerosis

Low density lipoprotein (LDL) plays a central role in atherosclerosis and is the permissive factor that allows other risk factors to operate [14]. The efficacy of LDL cholesterol-lowering in reducing cardiovascular morbidity and mortality, and also all-cause mortality, is well established [91]. Atherosclerotic lesion development begins with the internalisation of circulating LDL into monocyte-derived macrophages in the arterial wall. Macrophage uptake of unmodified LDL is slow, and it must undergo atherogenic modification by oxidation and / or glycation before uptake is rapid enough to excite foam cell formation and inflammation of the vessel wall [190].

Atherogenic Modification of LDL

The role of glucose (or species derived from glucose) in inducing these atherogenic modifications is particularly relevant to diabetes-associated atherosclerosis [292]. Hyperglycaemia induces the non-enzymatic glycation of proteins, resulting in the formation of advanced glycation end products (AGEs), which contribute to atherosclerosis through interactions with specific receptors in the vessel wall, including receptors for AGE (RAGE) [293]. Glycated LDL is present in the circulation under physiological conditions and at higher concentrations in diabetes and in people destined to experience myocardial infarction [205].

Glycation occurs either by direct attachment of glucose or indirectly after conversion of glucose to an oxidized analogue [294]. Glycation of LDL occurs in the lysine residue of apolipoprotein B (apo B), allowing it to act as a ligand for macrophage scavenger receptors [190]. Glycated LDL is taken up rapidly by macrophages in culture, and both oxidized and glycated LDL have been demonstrated in atherosclerotic lesions, where they exist at higher concentrations than in the circulation [25]. Thus ‘carbonyl stress’ may play a significant role in diabetes-associated atherosclerosis [295].

LDL modified by glycation is not cleared by the physiological LDL receptor [209]. Although its circulating concentration is several times higher than that of oxidized LDL, glycated LDL

remains relatively unstudied. Improved understanding of lipoprotein glycation might lead to therapeutic strategies to reduce circulating levels of glycated LDL where trials of antioxidant therapy in the prevention of atherosclerosis have been disappointing [296]. Here we assess the relationship between lipoprotein glycation and oxidation in patients from the Protection Against Nephropathy in Diabetes with Atorvastatin (PANDA) study.

***In vitro* Lipoprotein Glycation**

LDL glycation occurs *in vivo* at physiological glucose concentrations but requires supraphysiological concentrations *in vitro*. The ease of glycation *in vivo* remains largely unexplained [297]. Proposed mechanisms include modification by glucose metabolites and the presence of free oxygen radicals or transition metal cations *in vivo*. For example, if autoxidation of glucose to δ-gluconolactone occurs *in vivo*, it could offer a secondary pathway to lipoprotein glycation. This study aimed to investigate the effect of δ-gluconolactone, an oxidized (ketoaldehyde) derivative of glucose, on *in vitro* glycation of LDL [294].

Transition Metal Ions and Complications in Diabetes

Several prospective studies have found elevated serum copper concentrations to be associated with CVD, although it remains unclear whether copper directly affects atherogenesis or is simply a marker of associated inflammation [298]. The accumulation of reactive metals at sites of extensive glycation *in vivo* might have serious pathogological consequences, and atherosclerotic lesions have been shown to contain both copper and iron and in forms that catalyze free-radical formation [299]. Defective copper regulation has also been implicated in end-organ damage in diabetes, and treatment with a copper-selective chelator has recently been shown to improve cardiac function in diabetic rats [300]. The mechanisms by which hyperglycaemia leads to tissue copper excess remain unresolved, but dysregulation of the metabolism of copper could be mediated by AGEs [301]. Treatment of diabetic rats with a copper chelator significantly reduced not only levels of copper but also AGEs and AGE-precursors [302]. Here we explore the effect of copper on *in vitro* lipoprotein glycation, and whether any effect can be attenuated by chelation using Triethylenetetramine (TETA), a highly-selective divalent copper chelator used in the treatment of Wilson's disease, but with potential therapeutic uses in diabetes [303].

High-density lipoprotein Functionality

High-density lipoprotein (HDL) cholesterol is an independent risk factor for CVD, and is superior even to LDL as a predictor of cardiovascular events [41]. Although HDL levels are a strong biomarker for assessing CVD risk, emerging evidence suggests that HDL functionality is more important than cholesterol cargo [12]. Potentially atheroprotective HDL activities appear to be reduced in patients with diabetes, and glycation may play a role in HDL

dysfunction. In this study we also consider the ability of HDL to impede LDL modification, and the effect of HDL oxidation / glycation on its functionality, through assessment of cholesterol efflux capacity.

Materials and Methods

Relationship between Glycated Apo B and Oxidized LDL

The PANDA study was a double-blind randomized clinical trial of Atorvastatin 10 mg/day (*n*=59) *vs*. 80 mg/day (*n*=60) in patients with type 2 diabetes mellitus and microalbuminuria or proteinuria, recruited from diabetes clinics in Greater Manchester. The study was approved by the local ethics committee and investigations carried out in accordance with the principles of the Declaration of Helsinki. Details of intervention, randomization, sampling and primary and secondary end-point outcomes has been described previously [304]. Total glycated apo B was determined by enzyme-linked immunosorbent assay (ELISA) kit from Glycacor kits (Exocell Inc. PA, USA) with intra- and inter-assay coefficients of variation of 3.5 and 14.9% respectively. Oxidized LDL was also assessed by ELISA (Mercodia, Sweden) with intra- and inter-assay coefficients of variation of 5.8 and 4.6% respectively. Plasma glucose was determined using an automated glucose oxidase method (Randox, Co. Antrim, UK).

Lipoprotein Isolation

LDL (density 1.019-1.063 g/ml) and HDL (1.063-1.21 g/ml) were isolated from pooled autologously donated human plasma samples (Central Manchester University Hospitals) using sequential preparative ultracentrifugation with aspiration of different density fractions. Ultracentrifugation procedures were performed in 11 x 34 mm^2 polycarbonate tubes (Beckman Coulter UK, High Wycombe, UK) with a Beckman TLA 120.2 fixed angle rotor (100,000 rpm; 435,680 x g for 5 hours) at 4^oC using a Beckman Optima TLX ultracentrifuge. Lipoprotein fractions were then dialysed overnight against pH 7.4, 10 mM Phosphate Buffered Saline (PBS) at 4^oC followed by sterile filtration (0.2 μm) to remove Ethylenediaminetetraacetic acid (EDTA) and potassium bromide, and stored at 4^oC under nitrogen for not more than 16h before glycation studies. Recovery of apo B in LDL fractions was 85 ± 5% (mean ± S.D.). The protein concentration in isolated lipoprotein fractions was measured by the bicinchonic acid (BCA; Pierce and Warner Ltd., Chester, UK) method using 0.4 mg/ml Bovine Serum Albumin (BSA; Merck, Darmstadt, Germany) as a standard, with incubations performed at 60^oC for 45 – 60 minutes.

***In vitro* Glycation**

Glycated apo B, total apo B and lipid peroxide concentrations were determined in the presence and absence of reactive sugars, glucose and copper, before incubation (zero time) and after incubation for up to 7 days at 37^oC. Isolated LDL was incubated under nitrogen in

airtight screw-capped 2 ml polypropylene sample tubes in a Gallenkamp Economy Size 1 Incubator (Gallenkamp, Leicester, UK), at a concentration of 1 mg/ml with glucose and copper at physiological concentrations (5 – 25 mmol/l and 1.25 – 5 μmol/l respectively) in PBS containing 0.01% chloramphenicol for 7 days [305]. These conditions allow minimal oxidation, but extensive glycation of apo B. Glucose, δ-gluconolactone and copper sulphate / copper chloride were supplied by the Sigma Chemical Co. (Poole, UK). All solutions were freshly prepared with deionized water (Milli Q system, Millipore-Waters). Copper-histidine solution was prepared as described [306]. Blanks consisted of lipoproteins in PBS without other additives.

Where required, mildly oxidized LDL was prepared by incubation of LDL (1mg/ml) with copper sulphate (10 mmol/l) in sodium PBS for 24h at 37°C. In experiments designed to assess the effect of HDL on *in vitro* glycation HDL was included in incubations at a concentration of 0.5 mg/ml. Where indicated, TETA was included in incubations at concentrations of 0.1 – 10 mg/l, to reflect therapeutic plasma concentrations of 4 – 5 μmol/l. The dosage used was based on those employed in clinical applications of TETA in the treatment of patients with Wilson's disease, and as an experimental therapy for diabetes [307]. We used TETA disuccinate because of its improved stability characteristics.

Following incubation with reactive sugars or glucose and copper, unbound glucose was removed by overnight dialysis at 4°C against 1 L of PBS (pH 7.4), and the protein concentration again determined. Glycated lipoproteins were then separated from non-glycated lipoproteins using *m*-aminophenylboronate affinity chromatography with Glycogel II test columns packed with immobilized boronic acid gel (Pierce Chemical Company, Rockford, IL) [187]. Each column was first equilibrated with 10 ml of ammonium acetate wash buffer (250 mmol/l ammonium acetate, 50 mmol/l $MgCl_2$, 500 mmol/l NaCl, 3 mmol/l NaN_3 and 0.1% (v/v) Tween-20, pH 8.5). 100 μl of each incubation mixture was applied to the top of each column and the non-glycated proteins were then eluted using 5 ml of ammonium acetate buffer before elution of glycated proteins using 3.9 ml of sorbitol buffer (200 mmol/l sorbitol, 500 mmol/l NaCl, 50 mmol/l Na_2EDTA, 100 mmol/l tris/HCl and 0.1% (v/v) Tween-20, pH 8.5) [308].

Our in-house high-sensitivity ELISA method (detection limit 4.6×10^{-6} g/l, with intra- and inter-assay coefficients of variation 5.1 and 8.5% respectively) was used to measure apo B in the second chromatography eluate [206]. This ELISA employs unconjugated and horseradish peroxidase (HRP)-conjugated goat anti-human apo B polyclonal antibodies (Abcam) as capture and detection antibodies respectively. The apo B standard (Sigma) was used in the concentration range of 0.006 – 0.06 mg/dl. The amount of bound analyte was detected with the HRP-substrate, *o*-phenylenediamine (Sigma) and the colour intensity measured at 490 nm using a Dynatech MR7000 plate reader. The extent of glycation in LDL

can be expressed as mg of glycated apo B in the bound eluate per mg of apo B applied to the boron affinity chromatography column. This method achieves good recovery (glycated apo B recovery > 90% and non-glycated apo B recovery > 88%), even after recycling chromatography columns up to five times.

Lipid Peroxides (LPO) formed during glycation were measured by a cholesterol oxidase colorimetric assay [309]. *N*-ε-carboxymethyl-lysine (CML) was measured by sandwich ELISA (MyBioSource Inc, USA), with intra- and inter-assay coefficients of variation of 7.5 and 9.5% respectively.

Following *in vitro* glycation, relative electrophoretic mobility (REM) of native or modified LDL (15 μg) was determined using electrophoresis at pH 8.6 in 0.05 M barbital buffer in 0.8% agarose gel. Gels were fixed in 100% methanol (1 minute), stained for 5-10 minutes with Fat Red 7B (Sigma-Aldrich), destained for 5-10 minutes with 70% methanol, and dried at 60°C. REM is defined as the ratio of the distances travelled by modified LDL and native LDL. Examination of the REM of modified LDL particles yields information on the overall net positive charge; less positively charged particles (associated with modification of apo B Arginine and Lysine residues) move further into the gel than native LDL.

Glycation of LDL and Foam Cell Formation

THP-1 cells (ECACC, Salisbury, UK) were differentiated into mature macrophages by incubation in serum-free RPMI-1640 media (Cambrex bioscience, Verviers, Belgium) containing 200 nmol/l phorbol 12-myristate 12-acetate (Sigma Chemical Co., Poole, UK) for 72h at 37°C. Differentiated macrophages were incubated with serum-free RPMI-1640 media containing 4 mmol/l glutamine and 1% (v/v) Penicillin / Streptomycin (both Cambrex bioscience, Verviers, Belgium), 1% non-essential amino acids (Lonza, Verviers, Belgium) and 10% Lipoprotein deficient serum with freshly isolated or glycated LDL (50 μg/ml) for 48h followed by an overnight equilibrium period in media containing 1 mg/ml bovine serum albumin (BSA; Sigma Chemical Co., Poole, UK), before cell lysis with water for 20 minutes at 4°C. Foam cell formation was evaluated by oil red O (Sigma Chemical Co., Poole, UK) staining and viewed using an Olympus CKX41 inverted microscope (magnification x 100). Free cholesterol, cholesterol esters and total cholesterol were assayed in the cell lysate after centrifugation at 353 x g for 5 minutes to remove cellular debris. Free cholesterol and total cholesterol were measured by enzymatic photometric cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) methods as described [310] and using a Cobas Mira autoanalyser (Horiba ABX, Northampton, UK). Cholesterol esters were calculated by subtracting free cholesterol from total cholesterol.

Glycation and HDL Functionality

Paraoxonase-1 (PON1) activity was determined by a semi-automated microtitre plate method using paraoxon (*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphate) as a substrate and read by spectrophotometer at 405 nm [311]. Intra- and inter-assay coefficients of variation were 3.5 and 2.7% respectively.

Cholesterol efflux was measured by a method based on that described by de la Llera-Moya *et al* [312]. Briefly, J774A.1 cells were incubated with radiolabelled cholesterol. These cells were then incubated with HDL isolated by sequential preparative ultracentrifugation for 4 hours. After incubation, the cell media were collected and cells washed with PBS and dissolved in 0.5 ml 0.2N sodium hydroxide to determine radioactivity. Cellular cholesterol efflux is expressed as the fraction of radioactivity in the medium over the total radioactivity in the cells and medium. The intra-assay coefficient of variation was 3.9%.

Data Analysis

Data are expressed as mean ± SD of at least three separate experiments. Statistical analysis was performed using Statistical Package for Social Sciences software (SPSS, Illinois, USA). Statistical differences were sought using Student's t-test or one way ANOVA to determine changes in glycated-LDL for the *in vitro* studies. The percentage increase in glycated apo B after 7 days incubation with glucose was determined relative to the glycated apo B concentration before incubation. Significance was defined at the 5% level, with $P < 0.05$ assumed to be statistically significant. Spearman rank correlation was used to assess the association between LDL glycation and oxidation parameters.

Results

Correlation of Oxidized LDL and Glycated Apo B

There is a positive correlation between oxidized LDL and glycated Apo B, which becomes stronger following treatment with Atorvastatin (Figure 4.1). There was also a positive correlation between glucose and glycated apo B ($r = 0.27$, $P < 0.05$) following treatment though not at baseline.

To further investigate the relationship between oxidation and glycation, ease of apo B glycation *in vitro* was compared between freshly isolated LDL and mildly oxidized LDL. Glycated Apo B may be more susceptible to oxidation, but mildly oxidized apo B appears to be, if anything, less susceptible to *in vitro* glycation ($P = 0.07$).

***In vitro* Glycation with Glucose / δ-gluconolactone**

Incubation of LDL with increasing concentrations of glucose for up to 7 days produced a dose-dependent increase in LDL glycation (Figure 4.2). As previously reported, physiological concentrations of glucose are not sufficient to glycate LDL *in vitro*. Non-significantly

increased *in vitro* glycation was observed in reaction mixtures containing glucose concentrations of 10, 20 and 25 mmol/l (*P* = 0.47, 0.08 and 0.06 respectively). Incubation with 30, 50 and 80 mmol/l did significantly increase *in vitro* glycation (*P* 0.01, 0.04 and 0.04 respectively). Increasing glycation was also observed as the duration of incubation with glucose was increased from 0 to 7 days.

We also observed non-significant generation of LPO during *in-vitro* glycation experiments. LPO levels increased in parallel with glycation during incubation with both glucose and δ-gluconolactone, even in experiments conducted under nitrogen with no source of oxygen or other free-radical-generating substances (other than glucose) [206]. No further LPO formation occurred over 7 days and there was no change in electrophoretic mobility in the absence of glucose, suggesting that lipid peroxidation is dependent on glycation in this experimental system.

δ-gluconolactone, an oxidized analogue of glucose, rapidly glycates LDL *in vitro* [294]. *In vitro* Glycation can be achieved at lower concentrations (greater than 10 mmol/l) compared to glucose, and with shorter incubations. Again, increasing glycation is observed with increasing duration of incubation, but significant glycation can be achieved within 5 hours. More prolonged incubation periods (greater than 72 hours) are associated with significant protein degradation. δ-gluconolactone is a more potent oxidizing agent than glucose, with LPO increasing from 83.37 nmol/ml to 149.07 nmol/ml when LDL is incubated with 12.5 mmol/l δ-gluconolactone for just 5 hours (*P* < 0.001). Incubation with δ-gluconolactone is thus associated with more significant glycation and LPO generation than incubation with glucose.

***In vitro* Glycation and Foam Cell Formation**

Cellular total cholesterol, free cholesterol and cholesterol ester levels were increased (all measures *P* < 0.001) in THP-1 macrophages exposed to glycated LDL compared with those exposed to native LDL (Figure 4.3). Increased foam cell formation was observed with glycated LDL compared to native LDL.

***In vitro* Glycation in the presence of Copper**

Glycation *in vitro* occurs more readily in the presence of physiological concentrations of copper. Incubation with 25 mmol/l glucose and 5 μmol/l copper sulphate for 3 days increased glycated apo B from 1.83% to 3.89% (*P* < 0.0001). Similarly, incubation with glucose alone (20 mmol/l) does not significantly increase *in vitro* glycation, but addition of 5 μmol/l copper sulphate does (*P* < 0.05). Incubation with copper sulphate in the absence of glucose does not significantly increase *in vitro* glycation.

We have confirmed this effect with more physiological glucose concentrations across a range of physiological copper concentrations (1.25 – 10 μmol/l) (Figure 4.4) and also demonstrated that supraphysiological concentrations of copper (20 μmol/l) overwhelm the experimental system. Incubation with 10 mmol/l glucose and 1.25 μmol/l and 2.5 μmol/l copper sulphate for 3 days significantly increased the proportion of glycated apo B (*P* = 0.003 and 0.01 respectively).

We also compared glycation and oxidation in the presence of copper chloride (Figure 4.5) and the more physiological copper histidine, and assessed the effect of physiological concentrations of ferrous salts. The most consistent effects on *in vitro* glycation were observed for copper concentrations commonly seen *in vivo*, with copper sulphate and copper chloride at concentrations greater than 10 mmol/l associated with reduced *in vitro* glycation across a range of glucose concentrations.

Similar effects were observed in the presence of copper sulphate, copper chloride and copper-histidine complex, but not ferrous chloride or ferrous sulphate. Incubation with all copper complexes was associated with increased *in vitro* glycation, but effects with ferrous chloride and ferrous sulphate at 1.25 – 2.5 μmol/l were less significant than those observed in the presence of copper chloride, copper histidine or copper sulphate at equivalent concentrations. When added to reaction mixtures containing LDL and glucose (10mmol/l), copper sulphate (1.25 μmol/l) increased *in vitro* glycation by 18.4% (*P* < 0.05), copper chloride (1.25 μmol/l) by 32.9% (*P* < 0.01), copper histidine (1.25 μmol/l) by 30.3% (*P* < 0.01), ferrous sulphate (1.25 μmol/l) by 13.6% (*P* = NS), and ferrous chloride (1.25 μmol/l) by 21.0% (*P* = NS).

Electrophoretic mobility of LDL was not significantly enhanced compared to native LDL following non-enzymatic glycation (Table 4.1). Similar effects were detected with the incubated controls, consistent with lipid peroxide induced modification. These minor changes were copper-independent and consistent with minor modification of Lys and Arg residues. Unlike purely glycated lipoproteins, AGE-modified lipoproteins have significantly increased electrophoretic mobility [313].

The highly-selective divalent copper chelator TETA appears to impede the *in vitro* glycation of apo B observed with incubations of glucose and copper chloride (Figure 4.6), and copper sulphate, though not that observed with copper histidine. Addition of TETA at doses as low as 0.1 mcg appeared effective (*P* = 0.08). As expected, this effect is specific for copper catalyzed reactions, and no effect was seen in reaction mixtures containing LDL and glucose alone or reaction mixtures containing ferrous salts.

As expected, addition of TETA significantly reduced LPO production in reaction mixtures containing copper. LPO formation was reduced with doses of TETA as low as 0.1 mcg (P = 0.03).

As shown above, AGE-modified lipoproteins have significantly increased electrophoretic mobility, but this was significantly reduced by the addition of TETA to reaction mixtures containing TETA (Table 4.2). This effect was specific for copper-catalyzed reactions.

Importantly, delayed addition of TETA did not reduce modification of LDL, with no reduction in expected glycated apo B or LPO formation.

N-ε-carboxymethyl-lysine (CML) is a major AGE, said to form via autoxidative glycation or glycoxidation, which involves the reaction of glucose with proteins, catalyzed by transition metals such as copper [314].

In keeping with the results for glycated apo B and LPO, *in vitro* glycation with physiological concentrations of glucose alone did not increase CML formation at 72h (Figure 4.7); addition of physiological concentrations of copper does increase CML formation (P = 0.09), an effect which is attenuated by the inclusion of HDL in reaction mixtures. Similarly, the effect of copper chloride on CML production is attenuated by the addition of TETA (P = 0.02).

Effect of Ascorbate on *in vitro* Glycation

Given the common usage of ascorbate in *in vitro* glycation systems, we examined the effect of ascorbate on *in vitro* glycation at a range of concentrations, demonstrating that its antioxidant / antiglycative effect is only apparent at higher concentrations (Figure 4.8), concentrations significantly higher than those commonly achieved in clinical trials of antioxidant therapy [296], and that low concentrations may actually add to glycation.

High concentration (50 mmol/l) ascorbic acid significantly decreased *in vitro* glycation (P = 0.02) compared to incubations without vitamin C. For concentrations more likely to be encountered with vitamin C supplementation, low concentrations (50 μmol/l) were associated with significantly increased *in vitro* glycation (P = 0.03), but no significant difference was seen with concentrations of 100 μmol/l; concentrations of 200 μmol/l reduced glycated apo B by 18.8 ± 14.6% (P = 0.01).

High-dose (50 mmol/l) ascorbic acid significantly reduced lipid peroxide induced changes in REM (P = 0.02), in addition to reducing REM related to oxidation and autoxidative glycation / glycoxidation in the presence of transition metal ions ($P \leq 0.01$). Concentrations more consistent with those achieved in clinical trials (typical recommended doses range from 60 –

1000 mg/day, where doses of 1000 mg/day achieve plasma concentrations of ~150 μmol/l) do not have any significant effect on LPO generation or REM.

In vitro antioxidant effects are also observed with addition of butylated hydroxytoluene (BHT) at a range of concentrations. Incubation with 40 mMol BHT reduced REM in the presence of glucose and copper sulphate by almost 30% ($P < 0.0001$).

Effect of HDL on *in vitro* Glycation

The increase in glycated apo B observed on incubation of LDL with glucose was significantly reduced in the presence of HDL. This effect was more evident with higher glucose concentrations, but across all reaction mixtures incubation with HDL (0.5 mg/ml) was associated with a 12.5% reduction in glycated apo B formation *in vitro* ($P = 0.002$). HDL was as effective as TETA in impeding *in vitro* glycation in the presence of glucose and copper chloride (Figure 4.9).

The increase in LPO during LDL glycation was also largely abolished by addition of HDL. CML formation was only reduced (from 107.18 ± 36.36 to 57.38 ± 4.73 pg/ml, $P = 0.02$) by inclusion of HDL in reaction mixtures containing higher concentrations of glucose (50 mmol/l).

This effect was evident both for incubations with glucose and incubations with δ-gluconolactone (Figure 4.10). Here incubation with 12.5 mmol/l δ-gluconolactone for 5 hours (n=6) increased glycated apo B from 1.83% to 2.44% ($P = 0.0005$), but addition of HDL prevented glycation of apo B ($P < 0.001$). Addition of HDL also offers some protection against oxidation by δ-gluconolactone, with LPO reduced to 129.98 nmol/ml ($P = 0.22$).

Addition of HDL to reaction mixtures significantly reduces electrophoretic mobility in the presence of glucose; the reduction in mobility in the presence of transition metal ions did not reach significance.

Further (indirect) evidence for the antioxidant effect of HDL might be drawn from measurement of glucose by the glucose oxidase method in reaction mixtures prior to its removal by dialysis. Measured glucose concentrations are consistently lower in incubations containing HDL ($P = 0.17$).

Effect of Glycoxidation on HDL Functionality

In vitro glycation impairs HDL functionality, as assessed by PON1 activity and cholesterol efflux capacity (Figure 4.11). HDL is considered to be more resistant to *in vitro* glycation than LDL, but incubation of HDL with 10 mmol/l δ-gluconolactone for just 5h impairs PON1 activity (45.29 ± 31.27 nmol/min/ml *vs.* 46.8 ± 32.18 nmol/min/ml, $P = 0.06$). Incubation of

HDL with glucose also impairs cholesterol efflux capacity in a dose-dependent manner (P = 0.01 for glucose 80 mmol/l), an effect further enhanced by the presence of copper (P = 0.04 for glucose 10 mmol/l and copper chloride 1.25 μmol/l, P < 0.001 for glucose 80 mmol/l + copper chloride 1.25 μmol/l). This effect of glycation on cholesterol efflux capacity is also evident when efflux is assessed using apo B-deplete serum (P = 0.05 for glucose 10 mmol/l).

Discussion

LDL Quality in Diabetes

Atherosclerosis progression is associated with high levels of LDL and, more particularly, small dense LDL [315]. Although LDL levels are frequently normal in patients with diabetes, hyperglycaemia or other metabolic derangements may alter lipoproteins to forms that promote atherogenesis [316]. Increased atherogenicity of small dense LDL may be partly linked to non-oxidative modifications of apo B [317]. Small-dense LDL is more readily glycated than larger more buoyant LDL both *in vivo* and *in vitro*, possibly because a higher proportion of the apo B molecule is exposed to glucose [318]. *In vivo*, the longer circulating half-life of small-dense LDL compared with more buoyant LDL may also offer more opportunities for glycation [319].

Glycated LDL is not cleared by the physiological LDL receptor [209]. Both oxidized and glycated LDL are taken up via scavenger receptors in the arterial wall, loading macrophages with cholesterol and cholesteryl esters [320]; this is believed to be the first step in foam cell formation and the subsequent development of atherosclerosis [321].

Atherogenic Modification by Oxidation

Activation of oxidative stress by hyperglycaemia plays a major role in the pathogenesis of complications in diabetes [322]. The extent of this stress is a consequence of both the degree of prolonged hyperglycaemia and acute glucose fluctuations [323]. The evidence implicating lipid peroxidation and oxidative modification of LDL in atherosclerotic lesion development is well established [324], but non-enzymatic glycation of LDL may be just as important. LDL isolated from the plasma of patients with diabetes and coronary heart disease is more susceptible to oxidation *in vitro* than LDL from healthy subjects [325]. Hyperglycaemia in diabetes thus causes increased free radical production, including reactive oxygen species, by glucose auto-oxidation, non-enzymatic glycation of proteins, and the polyol pathway [326].

Atherogenic Modification by Glycation

Glycation of LDL thus leads both to reduced LDL clearance and increased susceptibility to oxidative modification [327]. Glycation of lipoproteins in diabetes was first reported over

30 years ago [328]. Non-enzymatic glycation comprises a series of reactions that include the covalent binding of reducing sugar aldehyde or ketone groups to reactive protein free amino groups, forming unstable Schiff bases. These Schiff bases undergo rearrangements to yield more stable Amadori products, whose reactive free carbonyl groups are responsible for some of the biological consequences of glycation [329]. Several groups have confirmed that Schiff bases or Amadori products generate reactive oxygen intermediates [330]. Crosslinking and subsequent degradation of proteins forms AGEs [331].

Although the relationship between glycated LDL and oxidized LDL, and the significance of glycation versus the two facets of glycoxidation remain unclear, it seems that glycated LDL shares some characteristics of oxidized LDL [332]. Glycation can be induced not just by reactive sugars such as glucose, but also by its α-oxoaldehyde metabolites; these intermediates can be derived from glucose degradation or directly from Schiff bases, and also through other pathways such as the polyol pathway.

Cross-sectional and prospective studies have indicated that the micro- and macrovascular complications of diabetes mellitus are at least partially mediated through the accumulation of AGEs including the predominant *N*-ε-carboxymethyl-lysine (CML), and activation of RAGE, with downstream inflammatory cascades [333]. AGE levels also correlate with levels of oxidized LDL [334].

The literature related to *in vitro* lipoprotein modification often uses the term glycation quite loosely, but the effects of early and late glycation can differ. *In vitro* lipoprotein modification by early glycation alone cannot be achieved without metal chelating antioxidants such as EDTA, and incubation under nitrogen to reduce oxygen exposure [335]. Increased electrophoretic mobility and AGE formation are consistent with more advanced (late) glycation.

Atherogenic Modification by Glycoxidation

The combination of glycation and oxidation observed *in vivo* is termed glycoxidation. Even when molecular oxygen and oxygen free radical-generating processes are absent, *in vitro* glycation is generally accompanied by lipid peroxide generation. Thus, a degree of LDL oxidation is probably inevitable during glycation [305].

Glycation can be induced by reactive sugars, such as glucose and its α-oxoaldehyde metabolites. These α-oxoaldehydes can be derived from glucose degradation and directly from unstable Schiff bases early in glycation, and also through other pathways, such as the polyol pathway in which α-oxoaldehyde intermediates are derived from fructose or the oxidation of polyunsaturated fatty acids [336]. Glycation is therefore itself an oxidative process [337]. AGE formation may be a consequence of hydroxyl radical-mediated Amadori

product fragmentation in glycated proteins, or of glycation, transition metal binding, and hydroxyl radical generation in the presence of hydrogen peroxide [338].

This study demonstrates that δ-gluconolactone, an oxidized analogue of glucose, is a potent glycating agent *in vitro*. δ-gluconolactone has a higher affinity for LDL than glucose itself. This increased chemical reactivity probably results from additional carbonyl function. These results demonstrate that oxidized monosaccharides can also non-enzymatically glycate lipoproteins and therefore support the hypothesis of autoxidative glycation. Further investigation of autoxidative glycation and glycoxidation is necessary to increase our understanding of the relative contribution of glycative and oxidative stresses in the pathogenesis of diabetic complications [294].

AGEs are in fact glycoxidation products formed by sequential glycation and oxidation reactions, and transition metal ions are potent catalysts of their formation *in vitro* [339]. The primary difference between autoxidative glycation and glycoxidation is the sequence of the reactions involved [294]. In glycoxidation, oxidative reactions follow the attachment of glucose to proteins, whereas autoxidative glycation results from the glycation of proteins by oxidized species formed by the oxidation of glucose [340]. Autoxidative glycation *in vivo* likely involves transition metal ion-catalyzed autoxidation of monosaccharides such as glucose to produce reactive ketoaldehyde analogues (which form ketoamine adducts with proteins) and superoxide and hydroxyl radicals, which cause fragmentation of proteins and LDL peroxidation [341].

Increased electrophoretic mobility was observed with glycated, oxidized and glycoxidized LDL, in keeping with previous reports [209], probably a consequence of the increased negative charge associated with the modification of lysine amino groups.

Transition Metal Ions

Glycation and oxidation have been shown to be so interdependent that it is beneficial to study the two mechanisms concurrently as possible causes of copper-induced damage [314].

Studies implicate an interaction between serum copper and chronic hyperglycaemia in the mechanism by which diabetes causes vascular damage. Free divalent copper, Cu^{2+}, is the most redox-active transition metal ion *in vivo* and increased circulating levels can cause tissue damage by generating highly-reactive hydroxyl radicals [342].

Several aspects of copper metabolism are altered in diabetic subjects, and isolated measurements of serum copper or caeruloplasmin are unlikely to be informative [343]. There is some evidence that chronic hyperglycaemia can damage the copper-binding

properties of both caeruloplasmin and albumin. The released bound copper then appears to participate in a Fenton-type reaction to produce hydroxyl radicals [344].

It has been proposed that AGEs on lipoproteins may exacerbate local oxidative damage by binding redox-active transition metal ions [345]. CML in particular acts as an endogenous copper chelator [346]. The binding of redox-active divalent copper by CML-rich proteins both *in vitro* and *in vivo* raises a number of important questions [338]. CML bound copper may contribute to the systemic excess of copper reported in diabetes [347].

Copper is an essential trace element and is vital to a number of enzymes [348]. Superoxide dismutase (SOD) scavenges superoxide radical in the cytoplasm [349], where it exists as a dimeric protein with copper and zinc ions in its catalytic centre [350]. The catalytic function of SOD is dependent on copper redox chemistry at its active site [351]. Glycation of SOD results in time-dependent release of Cu^{2+} with reduced enzymatic activity, fragmentation of the enzyme and generation of a hydroxyl radical, but not in the presence of EDTA, suggesting involvement of metal ions in this reaction [350].

The interaction of copper with the apo B component of LDL appears to involve binding to histidine residues [352]. After the binding of copper to LDL, lipid peroxidation requires the reduction of Cu^{2+} to Cu^{+} [316]. Glucose reduces Cu^{2+} to generate Cu^{+}, which reacts faster with lipid hydroperoxides, but has a lower affinity to LDL [353]. This may reduce the susceptibility of LDL to oxidation by copper ions [316]. Glucose could thus serve as an antioxidant through effects on copper binding to LDL [354], which may prevent the formation of pro-oxidant radicals in LDL. Others showed that the susceptibility of LDL to Cu^{2+}-induced oxidation increases in the presence of glucose, which may be explained by the reduction of Cu^{2+} to Cu^{+}, which rapidly breaks down preformed lipid hydroperoxides to generate free radicals, thereby propagating lipid peroxidation [341].

Copper is more potent than iron in its ability to modify LDL *in vitro* [355]. Unlike copper, ferrous ions do not induce LDL oxidation in the absence of an external reducing agent, and Fe^{3+} is not reduced by the LDL particle [356]. This may be a consequence of the reduced affinity of Fe^{3+} for apo B compared to Cu^{2+} ions [357].

Potential Role of Copper Chelation

Chelation of metal ions may therefore be of particular importance in patients with diabetes mellitus [299]. In conjunction with therapies aimed at decreasing hyperglycaemia, the use of compounds that can remove systemic copper has been proposed as a therapy for the diabetic complications. TETA is a selective copper-chelator that binds free copper, thus suppressing copper-catalyzed reactions of reactive oxygen species, to generate hydroxyl

radicals in the extracellular matrix [343]. TETA dihydrochloride was previously shown to prevent albuminuria and heart failure in diabetic rats, and was here investigated as a potential interventional treatment against atherogenic LDL modifications [358]. Interestingly, some medications commonly used in diabetes mellitus may also have chelating properties [359]. Chelation by TETA prevents copper binding to the LDL particle, maintaining it in the aqueous phase and protecting it from modification [357]. If TETA removes weakly bound copper ions, it may inhibit AGE formation and thus reduce copper-catalyzed glycoxidative damage [343]. TETA treatment activates SOD by increasing the supply of copper for incorporation into the enzyme via improved copper chaperone function, without changing the expression of SOD protein itself [351].

Clinical trials of copper chelation in patients with diabetes mellitus and CVD currently in progress may offer opportunities to replicate these findings and define the mechanisms of benefit [360].

Antioxidants

Vitamin C (Ascorbate) is a potent water-soluble antioxidant, scavenging reactive oxygen and nitrogen species [361]. *In vitro* data suggest that vitamin C acts as an antioxidant at high concentrations, but as a pro-oxidant at lower levels [362]. An important biological function of vitamin C is its interaction with redox-active transition metal ions, such as iron and copper [363]. Paradoxically, the reduction of transition metal ions by ascorbate may increase hydroxyl radical or lipid alkoxyl radical generation by reactions with hydrogen peroxide or lipid hydroperoxides [361]. Although this Fenton chemistry occurs readily *in vitro*, its relevance *in vivo* remains controversial [364]. Ascorbate's effects may also depend on when it is added to the experimental system [365]. For example, it acts as an antioxidant if added before the initiation of LDL oxidation by copper, but as a pro-oxidant if added to LDL that is already (mildly) oxidized [366].

Interestingly, *in vitro* studies have also shown that Vitamin C can slowly glycate proteins under oxidizing conditions, forming AGEs [367]. However, oxidation of ascorbate to dehydroascorbic acid was required for the glycation reactions to occur [363].

Other antioxidant systems, like HDL-associated PON1, may be more important in the protection of LDL against oxidation [368].

HDL Functionality

HDL prevents copper-induced generation of lipid peroxides in LDL in a time-independent manner [369]. HDL is more resistant to glucose-induced glycation than LDL, and can protect LDL against glycation as well as oxidation. Here we confirm our previous report that HDL impedes the glycation of LDL. The mechanism by which HDL interferes with LDL glycation

remains to be established [71]. We have previously reported that when LDL is glycated in our experimental system, where there is no external source of oxygen free radicals, small quantities of LPO are still generated [305]. Accordingly HDL also decreases glycation-induced accumulation of LPO on LDL. The increase in the REM of LDL was also ameliorated when HDL was present. HDL might interfere with LDL glycoxidation *in vitro* by chelating Cu^{2+} [370].

There is also mounting evidence to suggest that the anti-atherogenic properties of HDL are impaired in patients with diabetes [371]. Glycation of HDL takes place mainly on apolipoprotein AI, the major apolipoprotein in HDL, diminishing HDL receptor binding and its ability to stabilize ATP-binding cassette transporter [372]. This would be expected to impair reverse cholesterol transport.

Future Work

It will be interesting to explore the role of PON1 in glycoxidation, including direct effects on glucose α-oxoaldehyde metabolites such as δ-gluconolactone. We will investigate the conformational changes in lipoproteins induced by glycoxidation, and further explore effects of oxidation / glycation on HDL functionality. Large gaps remain in our understanding of the effects of hyperglycaemia on the molecular regulation of copper homeostasis, and the basis of its restoration by TETA treatment, but these results warrant further investigations into properties of AGE-copper complexes and their impact on the development of diabetic complications [301]. Alongside efforts to clarify these issues, we will investigate the effect of TETA on glycated apo B and oxidized LDL levels *in vivo*.

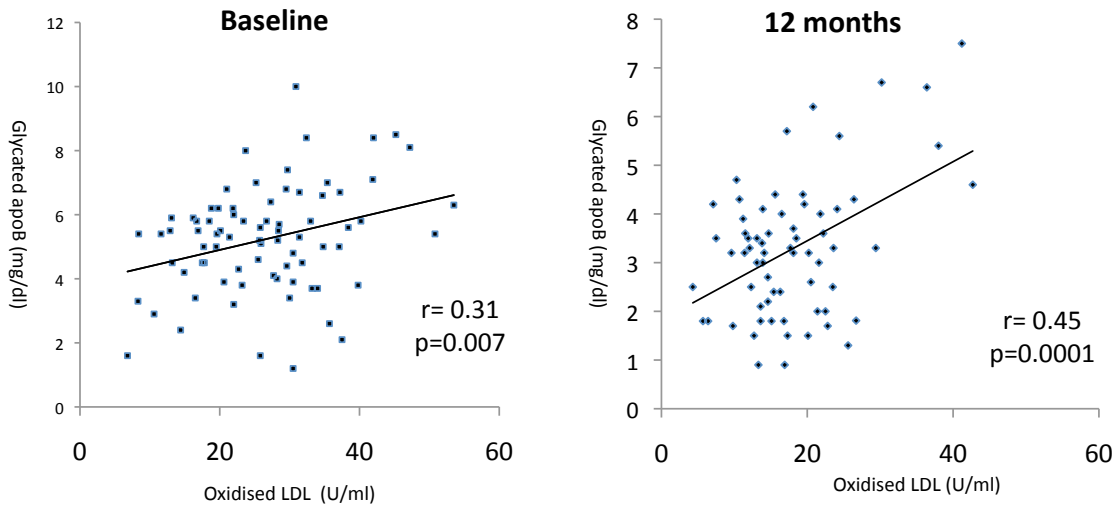

Figure 4.1: Relationship between Glycated apoB and Oxidized LDL in the PANDA study. *ApoB: Apolipoprotein B; LDL: Low-density lipoprotein; PANDA: Protection Against Nephropathy in Diabetes with Atorvastatin)*

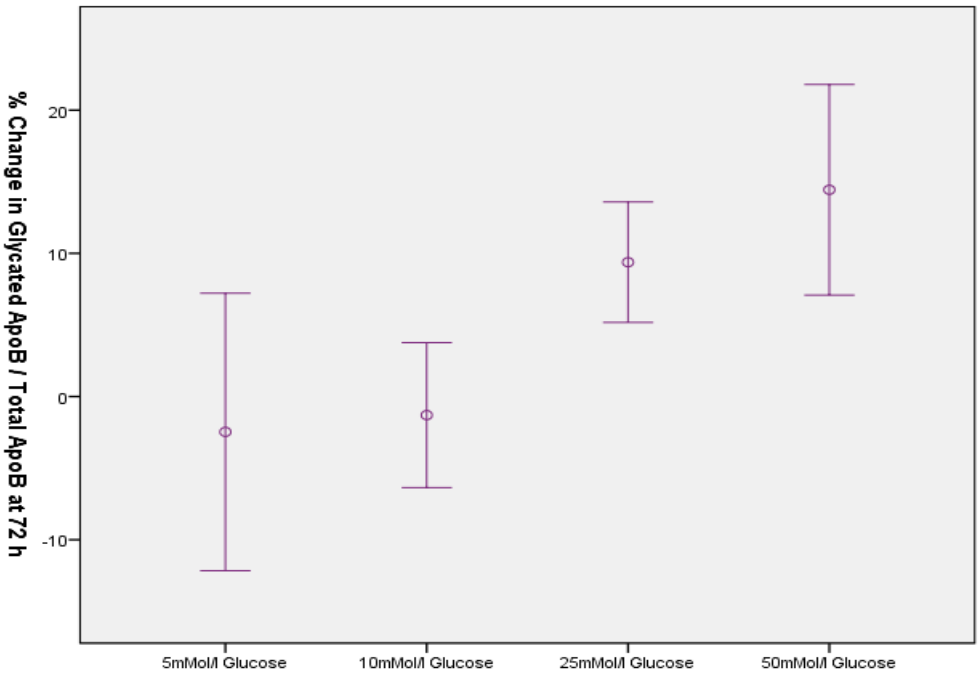

Figure 4.2: *In vitro* glycation requires supraphysiological glucose concentrations for longer (7 days) than the circulating half-life of LDL. *ApoB: Apolipoprotein B; LDL: Low-density lipoprotein*

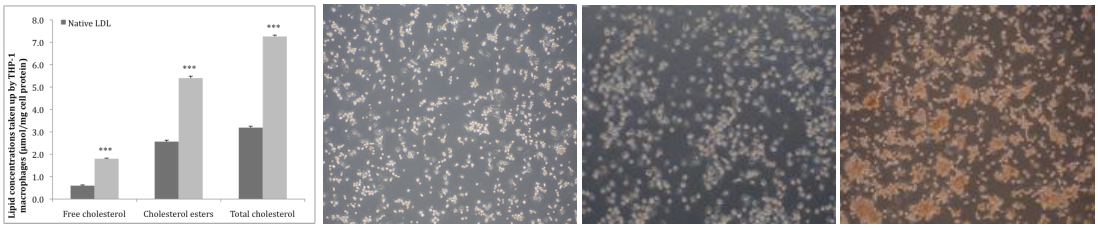

Figure 4.3: Lipid concentrations taken up by native and glycated LDL, and foam cell formation with Lipoprotein deficient serum, Native LDL, and Glycated LDL after 48h. *LDL: Low-density lipoprotein*

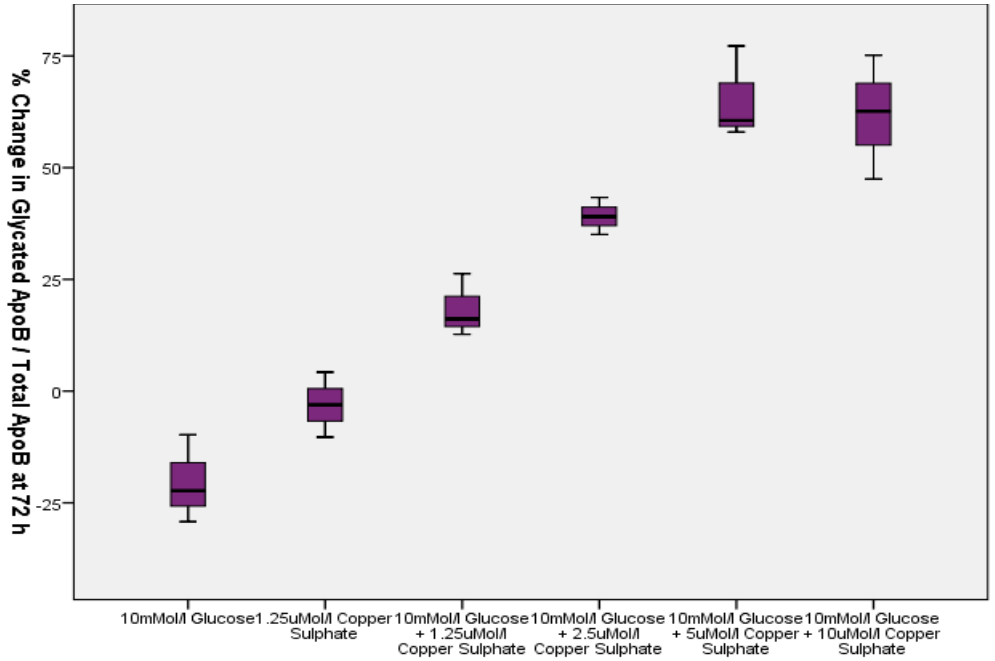

Figure 4.4: Effect of copper sulphate on *in vitro* glycation with glucose at 72h. *ApoB: Apolipoprotein B*

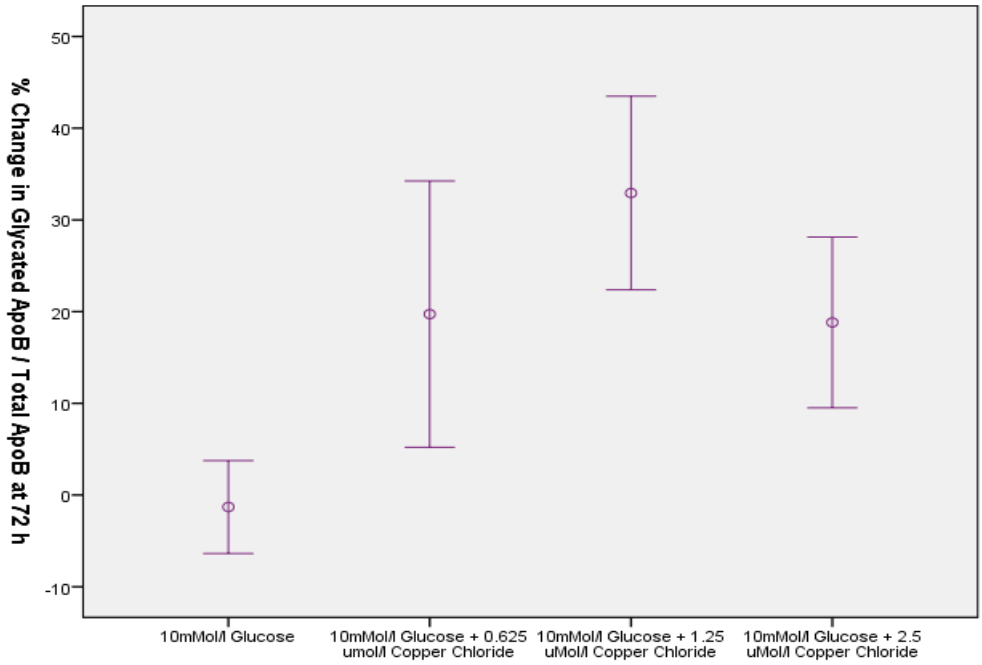

Figure 4.5: Effect of copper chloride on *in vitro* glycation with glucose at 72h. *ApoB: Apolipoprotein B*

Incubation Mixture	*REM (mean ± SD)*	*Incubation Mixture*	*REM (mean ± SD)*
LDL + Glu (10mmol/l) *(n = 14)*	1.0 ± 0.10 *(P = NS)*	LDL + Glu (10mmol/l) + $CuCl_2$ (0.625μmol/l) *(n = 5)*	1.02 ± 0.25 *(P = NS)*
LDL + Glu (20mmol/l) *(n = 9)*	1.04 ± 0.08 *(P = NS)*	LDL + Glu (10mmol/l) + $CuSO_4$ (1.25μmol/l) *(n = 3)*	1.35 ± 0.21 *(P < 0.0001)*
LDL + Glu (25mmol/l) *(n = 8)*	0.99 ± 0.13 *(P = NS)*	LDL + Glu (10mmol/l) + $CuCl_2$ (1.25μmol/l) *(n = 14)*	1.35 ± 0.34 *(P = 0.002)*
LDL + Glu (50mmol/l) *(n = 10)*	1.0 ± 0.14 *(P = NS)*	LDL + Glu (10mmol/l) + $FeSO_4$ (1.25μmol/l) *(n = 3)*	1.07 ± 0.07 *(P < 0.001)*
LDL + $CuCl_2$ (0.625μmol/l) *(n = 5)*	1.06 ± 0.20 *(P = NS)*	LDL + Glu (10mmol/l) + $FeCl_2$ (1.25μmol/l) *(n = 4)*	1.04 ± 0.08 *(P = NS)*
LDL + $CuSO_4$ (1μmol/l) *(n = 3)*	1.33 ± 0.27 *(P = 0.0001)*	LDL + Glu (10mmol/l) + $CuSO_4$ (2.5μmol/l) *(n = 3)*	1.42 ± 0.26 *(P < 0.0001)*
LDL + $CuSO_4$ (1.25μmol/l) *(n = 3)*	1.39 ± 0.21 *(P < 0.0001)*	LDL + Glu (10mmol/l) + $CuCl_2$ (2.5μmol/l) *(n = 3)*	1.41 ± 0.21 *(P < 0.0001)*
LDL + $CuCl_2$ (1.25μmol/l) *(n = 11)*	1.38 ± 0.34 *(P < 0.001)*	LDL + Glu (10mmol/l) + $FeSO_4$ (2.5μmol/l) *(n = 3)*	0.99 ± 0.07 *(P = NS)*
LDL + $FeCl_2$ (1.25 μmol/l) *(n = 3)*	1.03 ± 0.13 *(P = NS)*	LDL + Glu (20mmol/l) + $CuSO_4$ (1μmol/l) *(n = 3)*	1.31 ± 0.22 *(P < 0.0001)*
LDL + $CuSO_4$ (2μmol/l) *(n = 3)*	1.42 ± 0.30 *(P < 0.0001)*	LDL + Glu (20mmol/l) + $CuSO_4$ (2μmol/l) *(n = 3)*	1.38 ± 0.27 *(P < 0.0001)*
LDL + $CuSO_4$ (2.5μmol/l) *(n = 3)*	1.46 ± 0.24 *(P < 0.0001)*	LDL + Glu (20mmol/l) + $CuSO_4$ (4μmol/l) *(n = 3)*	1.42 ± 0.29 *(P < 0.0001)*
LDL + $CuSO_4$ (4μmol/l) *(n = 3)*	1.46 ± 0.37 *(P < 0.0001)*	LDL + Glu (20mmol/l) + $CuSO_4$ (5μmol/l) *(n = 6)*	1.19 ± 0.12 *(P < 0.0001)*
LDL + $CuSO_4$ (5μmol/l) *(n = 6)*	1.21 ± 0.15 *(P < 0.0001)*	LDL + Glu (20mmol/l) + $CuSO_4$ (8μmol/l) *(n = 3)*	1.41 ± 0.30 *(P < 0.0001)*
LDL + $CuSO_4$ (8μmol/l) *(n = 8)*	1.40 ± 0.31 *(P < 0.0001)*	LDL + Glu (25mmol/l) + $CuCl_2$ (1μmol/l) *(n = 3)*	1.70 ± 0.28 *(P < 0.0001)*
		LDL + Glu (25mmol/l) + $CuCl_2$ (2μmol/l) *(n = 3)*	1.76 ± 0.30 *(P < 0.0001)*
		LDL + Glu (25mmol/l) + $CuCl_2$ (4μmol/l) *(n = 3)*	1.83 ± 0.31) *(P < 0.0001)*

Table 4.1: REM (relative to LDL alone) for reaction mixtures. *REM: Relative electrophoretic mobility; LDL: Low-density lipoprotein; Glu: Glucose; $CuCl_2$: Copper*

chloride; $CuSO_4$: Copper sulphate; $FeCl_2$: Ferrous chloride; $FeSO_4$: Ferrous sulphate

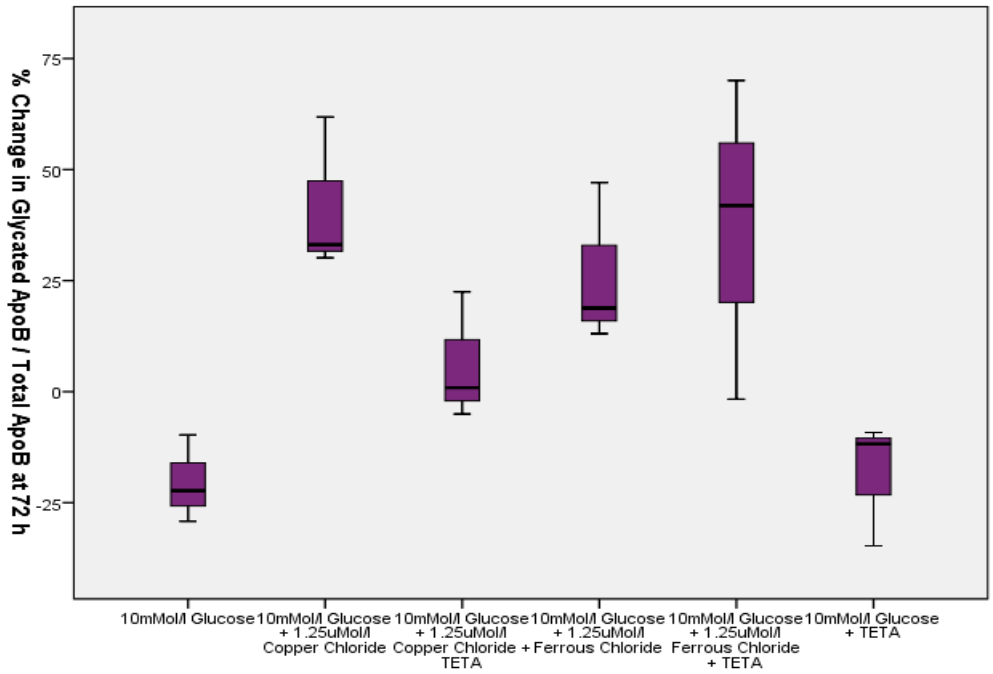

Figure 4.6: Effect of TETA on copper catalyzed *in vitro* glycation. *ApoB: Apolipoprotein B; TETA: Triethylenetetramine*

Incubation Mixture	*REM (mean ± SD)*	*Incubation Mixture*	*REM (mean ± SD)*
LDL + $CuCl_2$ (1.25μmol/l) + Glu (10mmol/l) + TETA 0.1mcg *(n = 5)*	0.92 ± 0.15 *(P = 0.02)*	LDL + Glu (10mmol/l) + TETA 10mcg *(n = 6)*	0.89 ± 0.12 *(P = NS)*
LDL + $CuCl_2$ (0.625μmol/l) + Glu (10mmol/l) + TETA 1mcg *(n = 5)*	0.83 ± 0.15 *(P = 0.02)*	LDL + Glu (50mmol/l) + TETA 10mcg *(n = 5)*	0.89 ± 0.14 *(P = NS)*
LDL + $CuCl_2$ (1.25μmol/l) + Glu (10mmol/l) + TETA 1mcg *(n = 10)*	0.85 ± 0.18 *(P = 0.0004)*	LDL + $CuCl_2$ (1.25μmol/l) + TETA 10mcg *(n = 6)*	0.87 ± 0.14 *(P = 0.004)*
LDL + $FeCl_2$ (1.25μmol/l) + Glu (10mmol/l) + TETA 10mcg *(n = 3)*	0.97 ± 0.25 *(P = NS)*	LDL + $CuCl_2$ (1.25μmol/l) + Glu (10mmol/l) + TETA 10mcg *(n = 6)*	0.86 ± 0.18 *(P = 0.004)*

Table 4.2: REM (relative to LDL alone) for reaction mixtures containing TETA. *P* values represent null hypothesis against equivalent mixtures without TETA. *REM: Relative electrophoretic mobility; LDL: Low-density lipoprotein; $CuCl_2$: Copper chloride; Glu: Glucose; TETA: Triethylenetetramine; $FeCl_2$: Ferrous chloride*

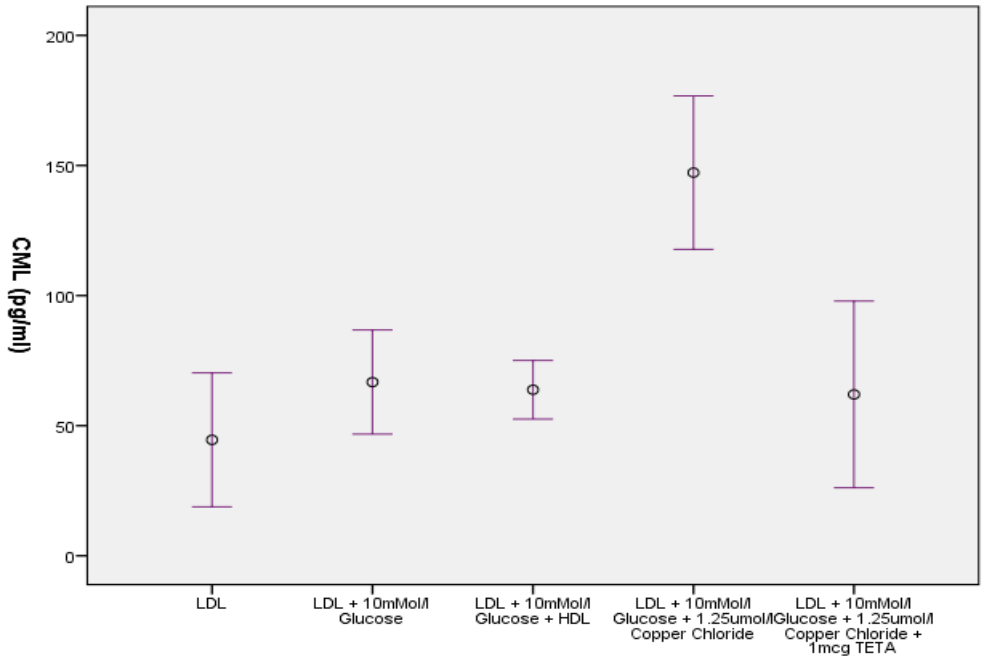

Figure 4.7: Formation of AGE in reaction mixtures. *CML: N-ε-carboxymethyl-lysine; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TETA: Triethylenetetramine*

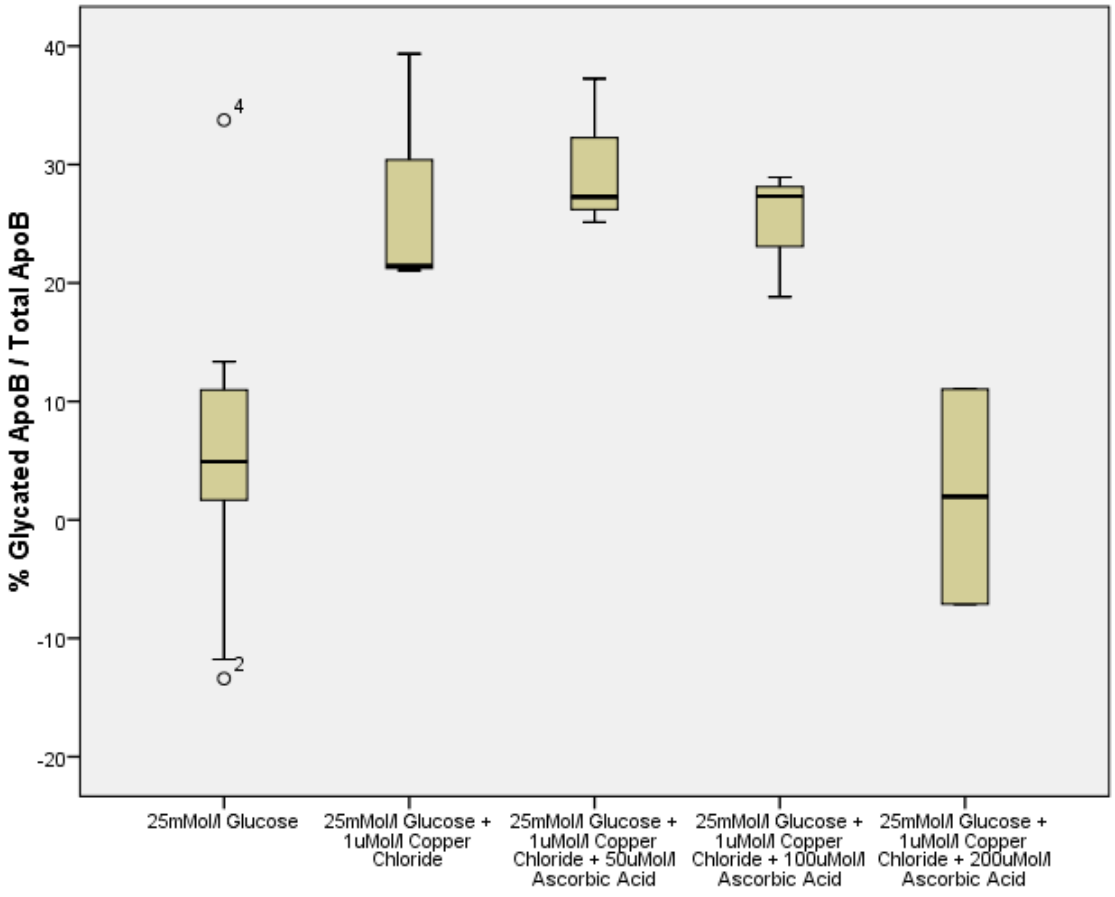

Figure 4.8: Effect of ascorbate on *in vitro* glycation. *ApoB: Apolipoprotein B*

Incubation Mixture	*REM (mean ± SD)*	*Incubation Mixture*	*REM (mean ± SD)*
LDL + Glu (25mmol/l) + $CuCl_2$ (1μmol/l) + Vit C (50μmol/l) *(n = 4)*	1.62 ± 0.13 *(P = NS)*	LDL + Glu (25mmol/l) + $CuCl_2$ (1μmol/l) + Vit C (200μmol/l) *(n = 4)*	1.57 ± 0.21 *(P = NS)*
LDL + Glu (25mmol/l) + $CuCl_2$ (2μmol/l) + Vit C (50μmol/l) *(n = 4)*	1.71 ± 0.20 *(P = NS)*	LDL + Glu (25mmol/l) + $CuCl_2$ (2μmol/l) + Vit C (200μmol/l) *(n = 4)*	1.63 ± 0.22 *(P = NS)*
LDL + Glu (25mmol/l) + $CuCl_2$ (4μmol/l) + Vit C (50μmol/l) *(n = 4)*	1.75 ± 0.20 *(P = NS)*	LDL + Glu (25mmol/l) + $CuCl_2$ (4μmol/l) + Vit C (200μmol/l) *(n = 4)*	1.67 ± 0.23 *(P = NS)*
LDL + Glu (50mmol/l) + $CuCl_2$ (1μmol/l) + Vit C (50μmol/l) *(n = 3)*	2.07 ± 0.14 *(P = NS)*	LDL + Vit C (50mmol/l) *(n = 3)*	0.72 ± 0.03 *(P < 0.0001)*
LDL + Glu (50mmol/l) + $CuCl_2$ (2μmol/l) + Vit C (50μmol/l) *(n = 3)*	2.11 ± 0.11 *(P = NS)*	LDL + Glu (20mmol/l) + $CuSO_4$ (1μmol/l) + Vit C (50mmol/l) *(n = 3)*	0.79 ± 0.08 *(P = 0.03)*
LDL + Glu (50mmol/l) + $CuCl_2$ (4μmol/l) + Vit C (50μmol/l) *(n = 3)*	2.18 ± 0.11 *(P = NS)*	LDL + Glu (20mmol/l) + $CuSO_4$ (2μmol/l) + Vit C (50mmol/l) *(n = 3)*	0.88 ± 0.16 *(P = 0.02)*
LDL + Glu (25mmol/l) + $CuCl_2$ (1μmol/l) + Vit C (100μmol/l) *(n = 4)*	1.48 ± 0.24 *(P = NS)*	LDL + Glu (20mmol/l) + $CuSO_4$ (4μmol/l) + Vit C (50mmol/l) *(n = 3)*	0.90 ± 0.15 *(P = 0.03)*
LDL + Glu (25mmol/l) + $CuCl_2$ (2μmol/l) + Vit C (100μmol/l) *(n = 4)*	1.64 ± 0.25 *(P = NS)*	LDL + Glu (20mmol/l) + $CuCl_2$ (8μmol/l) + Vit C (50mmol/l) *(n = 3)*	0.94 ± 0.17 *(P = 0.03)*
LDL + Glu (25mmol/l) + $CuCl_2$ (4μmol/l) + Vit C (100μmol/l) *(n = 4)*	1.66 ± 0.23 *(P = NS)*		

Table 4.3: REM (relative to LDL alone) for reaction mixtures containing ascorbic acid. *P* values represent null hypothesis against equivalent mixtures without ascorbic acid *(REM: Relative electrophoretic mobility; LDL: Low-density lipoprotein; Glu: Glucose; $CuCl_2$: Copper chloride; Vit C: Ascorbic acid; $CuSO_4$: Copper sulphate*

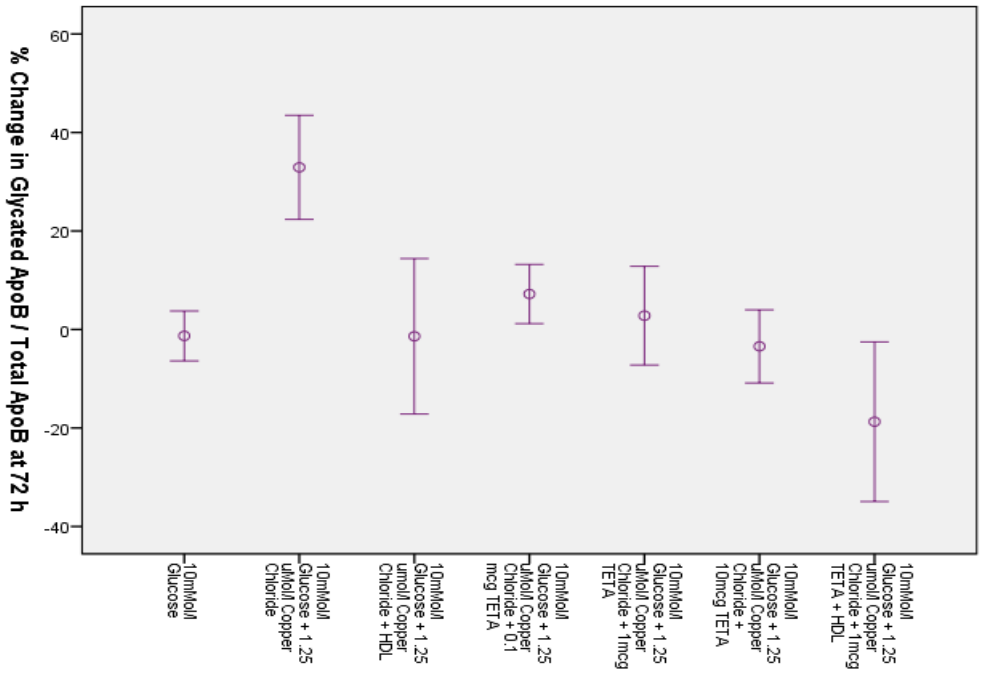

Figure 4.9: Effect of TETA and HDL on *in vitro* glycation in the presence of copper Chloride. *ApoB: Apolipoprotein B; HDL: High-density lipoprotein; TETA: Triethylenetetramine*

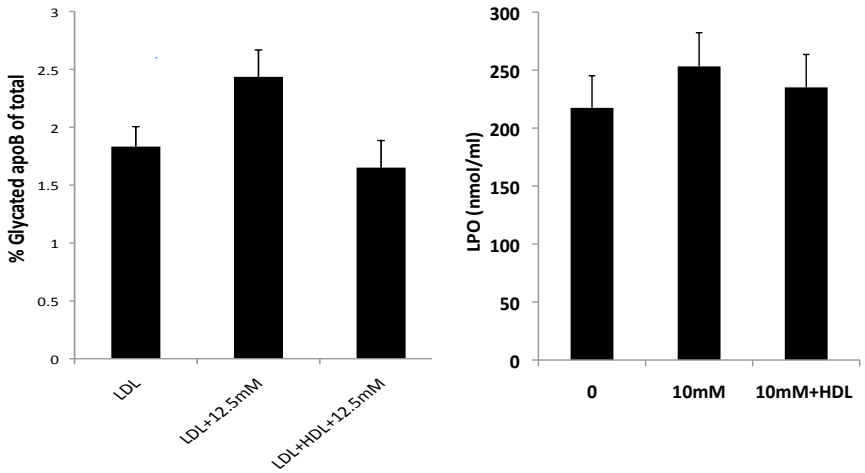

Figure 4.10: Effect of HDL on *in vitro* glycation and production of lipid peroxides for LDL with δ-gluconolactone. *ApoB: Apolipoprotein B; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; LPO: Lipid peroxides*

Incubation Mixture	*REM (mean ± SD)*	*Incubation Mixture*	*REM (mean ± SD)*
LDL + Glu (10mmol/l) + HDL *(n = 5)*	0.79 ± 0.15 *(P = 0.004)*	LDL + Glu (10mmol/l) + $CuCl_2$ (0.625μmol/l) + HDL *(n = 4)*	0.72 ± 0.22 *(P = NS)*
LDL + Glu (25mmol/l) + HDL *(n = 5)*	0.81 ± 0.14 *(P = 0.04)*	LDL + Glu (10mmol/l) + $CuCl_2$ (1.25μmol/l) + HDL *(n = 6)*	1.05 ± 0.40 *(P = NS)*
LDL + Glu (50mmol/l) + HDL *(n = 5)*	0.81 ± 0.15 *(P = 0.03)*	LDL + Glu (10mmol/l) + $CuCl_2$ (2.5μmol/l) + HDL *(n = 3)*	1.39 ± 0.31 *(P = NS)*

Table 4.4: REM (relative to LDL alone) for reaction mixtures containing HDL. *P* values represent null hypothesis against equivalent mixtures without HDL *(REM: Relative electrophoretic mobility; LDL: Low-density lipoprotein; Glu: Glucose; HDL: High-density lipoprotein; $CuCl_2$: Copper chloride*

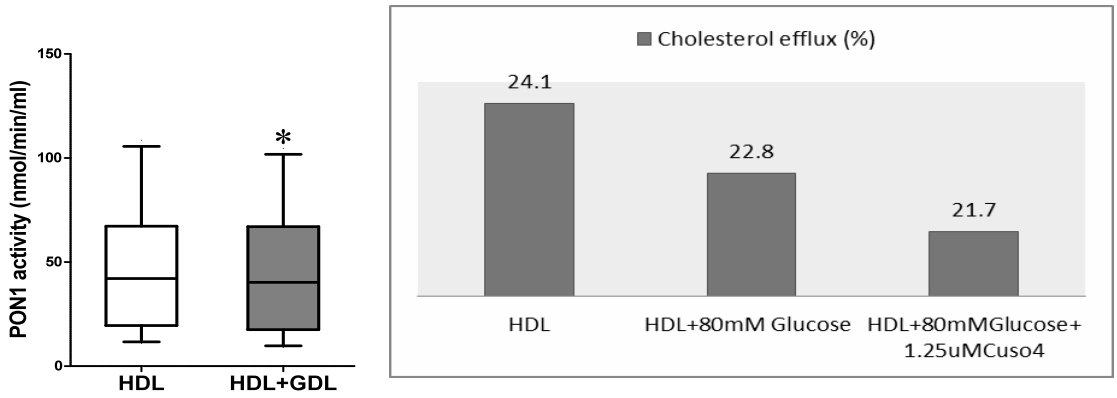

Figure 4.11: Effect of glycation on PON1 activity and Cholesterol Efflux from J774 macrophages to HDL. *PON1: Paraoxonase-1; HDL: High-density lipoprotein; GDL: δ-gluconolactone, Cuso4: Copper sulphate*

5. How HDL Protects Against Atherogenic Modification

Abstract

High-density lipoprotein (HDL) provides a pathway for the passage of lipid peroxides and lysophospholipids to the liver via hepatic scavenger receptors. Perhaps more importantly, HDL actually metabolizes lipid hydroperoxides preventing their accumulation on low-density lipoprotein (LDL), thus impeding its atherogenic structural modification. A number of candidates have been suggested to be responsible for HDL's antioxidant function, with paraoxonase-1 (PON1) perhaps the most prominent. Meta-analysis of clinical epidemiological investigations reveals a substantial association of low serum PON1 activity with coronary heart disease incidence independent of other risk factors including HDL cholesterol and apolipoprotein AI (apo AI). Transgenic animal models also indicate an antiatherosclerotic role for PON1. The therapeutic potential of PON1 should be recognised in preventing atherosclerosis and combatting infection and organophosphate toxicity. However, highly purified and recombinant PON1 do not retain their antioxidant properties.

Here I review the current evidence about how HDL impedes the oxidative and glycative atherogenic modification of LDL ans summarise the potential contributions of apolipoproteins, lipid transfer proteins, paraoxonases and other enzymes associated with HDL.

Introduction

Whilst atherogenesis is a complex process, macrophage-derived foam cell formation resulting from the uptake of circulating low-density lipoprotein (LDL) is of fundamental importance. There is no animal model of atheroma in which circulating cholesterol has not been raised usually by diet or genetic manipulation. In nations where the mean cholesterol of the population is less than 5 mmol/l, the incidence of premature mortality due to coronary heart disease is low [373, 374].

Despite this, foam cell formation with LDL is impossible to instigate *in vitro* due to insufficient monocyte-macrophage LDL receptor expression [375]. It was, however, discovered that experimental chemical modification of LDL permitted its rapid receptor-mediated uptake by monocyte-macrophages to form foam cells. This led to the identification of the scavenger receptors [375, 376] and opened new avenues of research to identify possible *in vivo* atherogenic modifications of LDL. Oxidation, glycation, and homocysteinylation have all been explored. Although clinical trials of chain-breaking antioxidants proved disappointing in the prevention of atherosclerotic cardiovascular disease [377], other systems which might oppose potentially atherogenic LDL modifications, including high-density lipoprotein (HDL) merit further attention.

It has been proposed that the HDL component that impedes oxidative modification of LDL is paraoxonase 1 (PON1) [368, 369, 378, 379], an enzyme located almost exclusively on HDL, initially studied because of its capacity to hydrolyze organophosphate pesticides and nerve gases. The Lipoprotein Research Group at the University of Manchester made important contributions to this notion; recent work has focused on glycation as an atherogenic modification of LDL and this too has led us back to PON1 [71]. Glycation of LDL is a more persistent and potentially important atherogenic modification as opposed to simple oxidation for which there are numerous metabolic safeguards [380].

Other HDL components have also been conjectured to be involved in preventing atherogenic LDL modification. The evidence for these emerges as probably less convincing than for PON1, although they may by coexisting in the same location provide a coordinated whole which provides a safe environment to receive lipid-derived noxious substances from not only other lipoproteins, such as LDL, but also from cell membranes during the passage of HDL through the tissue fluid, where it is the dominant lipoprotein species, and transporting them to the liver for elimination. Understanding these protective mechanisms might reveal important pathways which could be manipulated therapeutically to prevent atherosclerosis.

Atherogenic LDL Modification

The discovery that chemical modification of LDL by acetylation increases its affinity for macrophage scavenger receptors and reduces binding to the physiological LDL receptor led to a search for naturally occurring modifications which might have similar effects [375].

The ensuing hypothesis that lipid peroxidation products formed on LDL when subjected to attack by oxygen free radicals are responsible for changes in the apolipoprotein B100 (apo B) of LDL which alter its receptor binding preferences remains compelling despite the disappointing lack of effect of chain-breaking antioxidants [375-377]. Although a setback for the hypothesis, this lack of effect can perhaps be explained because once they have been oxidised in preference to say unsaturated lipids, the antioxidants themselves become pro-oxidant. Perhaps unsurprisingly given the safeguards against oxygen free radical damage, oxidized LDL is only found at low circulating concentrations, although it has been argued that higher levels might occur at sites where LDL is sequestered and this might include the arterial wall [376]. It has also been argued that antibodies to oxidatively modified LDL might be involved [375].

Nevertheless, it would be logical to consider that other naturally occurring modifications of LDL with similar receptor binding preferences to oxidatively modified LDL, but present more ubiquitously and at a higher concentration, might be critical to atherogenesis. Glycated LDL springs to attention for such a role. Using methods which I will also employ in patients with type 1 diabetes, colleagues in Manchester were surprised to discover using boron affinity chromatography and a sensitive apo B assay that glycated apo B was present in the circulation at relatively high concentrations of around 2–3 mg/dl in healthy people and at higher levels in type 2 diabetes and in hypercholesterolaemia [187]. This has been confirmed by immunoassays detecting epitopes unique to glycated LDL, and it has also been shown that atherogenic small dense LDL is more heavily glycated than other LDL subfractions *in vivo*, and is more susceptible to glycation *in vitro* [205, 305]. Glycated LDL also has a longer circulating half-life than unmodified LDL, and is removed from the circulation by route(s) not involving the LDL receptor [381]. Interestingly, statins also reduce circulating concentrations, likely by reducing LDL available to undergo glycation [205].

Homocysteine may also be atherogenic; thiolation of LDL free amino groups by homocysteine thiolactone increases its uptake by macrophages [382]. Interestingly the lactonase activity of PON1 will detoxify homocysteine thiolactone in addition to its role in preventing LDL oxidation (see later).

HDL Antioxidative Activity

Lipid hydroperoxides formed on LDL will migrate to its surface as a result of their greater hydrophilicity, facilitating their transfer to HDL [383]. This transfer can occur directly between lipoprotein phospholipid monolayers, but may be assisted by lipid transfer proteins (see Figure 5.1). HDL might thus provide a pathway for the passage of lipid peroxides and lysophospholipids to the liver via hepatic scavenger receptors. Although this mechanism undoubtedly exists, it was not realised until 1991 that HDL actually metabolises lipid hydroperoxides preventing their accumulation both on it and on LDL and thus impeding the atherogenic structural modification of LDL by the products of lipid peroxidation [368].

When HDL is incubated with LDL under oxidising conditions, the accumulation of lipid peroxides on LDL is decreased, but the concentration of lipid peroxides on HDL remains similar to that observed when HDL alone is oxidised (Figure 5.2) [379]. This effect of HDL is obvious within 3 h, by which time typically more than 50% of the lipid peroxidation of LDL, which would occur in the absence of HDL, has been prevented. This effect, which persists for several more hours, is not due to chain-breaking antioxidants or transition metal chelation, but due to enzymatic activity present on HDL [369, 378, 379]. It should also be noted that this anti-oxidative function of HDL is observed *in vitro* with similar protein concentrations of LDL and HDL; greater suppression of LDL oxidation might be expected when HDL concentrations exceed those of LDL as they do in the interstitial fluid. In fact, the accumulation of oxidised lipids in HDL likely results not only from their transfer from LDL but also from triglyceride-rich remnant particles and endothelial cells. The antioxidant effects of HDL have since been demonstrated by others in a variety of experimental systems and have proved relatively uncontroversial [60, 384].

HDL Antiglycative Activity

HDL can impede the modification of LDL by glycation *in vitro*, and this property of HDL is more marked with HDL obtained from people with higher serum PON1 activity [71]. In these experiments, to avoid lipid peroxidation as a confounding variable, external oxygen was eliminated as a source of free radicals. Under these conditions LDL is relatively resistant to glycation, such that supraphysiological glucose concentrations are required; even with very high concentrations of glucose it is not possible to glycate LDL to the extent found in many people with diabetes [380].

Oxidation appears to accompany *in vitro* glycation, and the process is best regarded as glycoxidation [206]. The lipid peroxidation of LDL that accompanies *in vitro* glycation is also impeded in the presence of HDL. Adduction of lipid peroxidation products to the ε amino groups of apo B lysine residues *in vivo* may render these groups more susceptible to combination with glucose. Thus, *in vivo* exposure of LDL to oxygen free radicals may predispose to glycation and explain the observed high levels of circulating glycated LDL.

The effect of HDL on glycation may thus be related to its anti-oxidative function. An alternative hypothesis is that the oxidized analog of glucose, gluconolactone, is more involved in apo B glycation, and that this step might be affected by PON1's lactonase activity.

Paraoxonase 1 (PON1)

PON1 is produced in the liver and circulates on HDL. There is a significant body of evidence to support a role for PON1 in atherosclerosis, and in particular against oxidation, not least its capacity to hydrolyze lipid hydroperoxides.

Colleagues at the University of Manchester previously demonstrated that the HDL fraction containing PON1 was most active in impeding Cu^{2+} induced lipid peroxide accumulation on LDL [369, 385]. It has since been suggested that it is not PON1 which is responsible for this effect, an argument supported by reports that more highly purified PON1 isolated from HDL and recombinant water-soluble variants of PON1 do not hydrolyze lipid peroxides [60, 386]. It is however exceptionally difficult to separate PON1 from other HDL components, such as apolipoprotein AI (apo AI) and phospholipase A2 (PLA2), without subjecting it to conditions which might affect its catalytic activity [387]. Similarly, the increased polarity of recombinant PON1 would be expected to compromise its ability to hydrolyze hydrophobic substrates [386, 388, 389]. More lipophilic recombinant PON1 might be expected to have improved functionality, but is more difficult to isolate, a factor which will prove important in the development of recombinant PON1 for therapeutic use [389]. The direct biochemical approach to determine the contribution of PON1 to the antioxidative activity of HDL clearly has its limitations, and evidence from other sources must be considered.

Epidemiological studies have consistently shown that PON1 activity is independently inversely associated with coronary events [390, 391]. A recent meta-analysis, which considered 47 such studies involving 9853 coronary heart disease cases and 11408 controls, reported that PON1 activity was 19% lower in patients suffering from coronary heart disease than in unaffected controls [391]. The largest single cohort study to date (Cleveland Clinic GeneBank study) involving 3668 patients following coronary angiography revealed a greater than two-fold risk of new cardiovascular events in the lowest compared with the highest quartile of serum PON1 measured as aryl esterase activity [392]. Other prospective studies have expanded on the negative correlation between PON1 activity and coronary heart disease by also reporting circulating levels of lipid peroxidation products, linking these to PON1 anti-oxidative activity [393, 394].

A number of medical conditions including diabetes mellitus, chronic kidney disease, familial hypercholesterolaemia and inflammatory arthritides are associated with both decreased

serum PON1 activity and increased cardiovascular risk [64]. PON1 activity is decreased in both type 1 and 2 diabetes [395, 396] and lower levels are associated with microvascular complications [395-397]. *PON1* and *PON2* genotype have been linked with susceptibility to develop diabetes [398], glycaemic control [399], and diabetic microvascular complications [395, 397, 400]. It has been suggested that this association reflects a role for oxidation in pancreatic β cell dysfunction or microvascular disease. Alternatively, it might reflect an ability of HDL / PON1 to prevent post-translational protein glycation. HDL rich in PON1 can impede the *in vitro* glycation of the apo B of LDL [71]. LDL glycation is a potentially atherogenic modification, and glycation of other proteins may be critical for the development of microvascular disease. The prospect that HDL and PON enzymes may be important in the prevention of post-translational protein glycation is thus intriguing [401].

HDL from avian species, which lacks paraoxonase activity, does not protect human LDL against lipid peroxidation [402]. Similarly, PON1 knockout mice are more susceptible to atherosclerosis and their HDL is less able to prevent the accumulation of lipid peroxides on human LDL [403], whereas transgenic rodent models expressing human PON1 are protected against atherosclerosis [404, 405]. In both the knockout and transgenic animals, these effects are achieved without any major change in lipoprotein metabolism. In contrast, knockout and transgenic animal models of *APO AI, lecithin: cholesterol acyl transferase (LCAT)* and *PLA2* have little discernible effect on atherosclerosis susceptibility, despite often substantial changes in lipoprotein metabolism [406-410].

PON1 has several genetic polymorphisms, the most extensively researched of which is the R192Q variant. This polymorphism has a substantial effect on PON1's capacity to hydrolyze paraoxon and homozygotes and heterozygotes for the R allele are more resistant to parathion (paraoxon is formed from this widely used organophosphate pesticide once it enters the body) than QQ homozygotes [411-413]. Other activities of PON1, such as phenyl acetate hydrolysis, which proceed at faster rates, are, however, unaffected by the R192Q polymorphism. In the case of the protective effect of HDL against LDL oxidation, HDL from 192QQ homozygotes is most effective in preventing the accumulation of lipid peroxides on LDL [379, 414], but this effect is small in comparison to the huge variation in serum PON1 activity. In two meta-analyses, relative cardiovascular disease risk was increased in people possessing a 192R allele as opposed to QQ homozygotes [411, 413]. In both of these, there was evidence of publication bias in favour of trials with a positive outcome and in both a more marked effect was evident in diabetes. The conclusion of the authors of one of the meta-analyses was that it did not exclude the possibility of an effect of the 192 polymorphism on CVD risk [411], whereas the other meta-analysis was interpreted as denying such an effect [413]. That serum PON1 activity (measured as paraoxon or phenyl acetate hydrolysis), representing the integral of genetic and acquired influences, is a

stronger determinant of coronary risk than genotype is, however, undeniable and amply confirmed by the recent findings of the Cleveland Clinic GeneBank study [392].

There is evidence that the parenteral administration of partially purified PON1 can ameliorate experimental atherosclerosis and a patent for PON1 as an antiatherogenic agent in humans exists [415]. Recombinant PON1 might also be used for this purpose, if its properties can be retained during isolation [386, 389]. Intraperitoneal injection of recombinant PON1 in mice increased HDL aryl esterase and lactonase activities, the resistance of HDL to oxidation and HDL-induced cholesterol efflux and decreased macrophage-mediated LDL oxidation [416]. Much of the current impetus for recombinant PON1 therapy is, however, on the one hand to protect people likely to be exposed to organophosphates or as an antidote in those who have been exposed [389, 417] and on the other hand, to combat serious infections [394].

Another development has been apo AI-mimetic peptides, some of which are orally active [418]. It might be thought that these mimic simply the actions of apo AI, but they create circulating lipid complexes which are a magnet for other HDL components such as PON1 [419]. We know that HDL is drawn to atheromatous lesions, where immunolocalization of both apo AI and PON1 occurs [420].

Paraoxonase 2 (PON2) and Paraoxonase 3 (PON3)

PON1 has two other family members, PON2 and PON3. *PON2* is almost exclusively expressed intracellularly, whereas PON3 is also associated with HDL, albeit in lesser quantities than PON1. The primary hydrolytic activity of PON3 is also as a lactonase [386]. *PON3* knockout mice are also more susceptible to atherosclerosis [405], but the reason for its evolutionary conservation is currently unclear.

Apolipoprotein AI (Apo AI)

Apo AI is essential both for the structure of HDL and the maintenance of the lipid environment in which enzymes such as PON1 and lecithin: cholesterol acyl transferase (LCAT) can operate [421]. It is thus an essential cofactor for both and is likely to have a major role in the antioxidant effects of HDL. Apo AI plays a central role in the redox inactivation of lipid hydroperoxides, which follows their transfer to HDL. It also creates a safe environment for the release of lysophospholipids and their subsequent transfer to the liver. Despite experimental evidence that lipid-protein complexes containing only apo A1 can protect LDL against oxidation [394], neither animal models nor human genetic disorders have provided convincing evidence that apo AI's anti-atherogenic effects are independent of changes in HDL levels [406, 407].

Apolipoprotein AII (Apo AII)

Apolipoprotein AII-containing HDL particles tend to be larger and possess less antioxidant activity than those with higher apo AI content [394]. There is evidence from both animal models and human studies to suggest that apo AII might actually suppress PON1 binding to HDL [422]. Mice expressing human apo AII and apo AI are more susceptible to atherosclerosis than those expressing apo AI alone. Delivery of the antioxidant capacity of HDL to vulnerable tissue sites may be fundamental to its function.

Other Apolipoproteins

Other apolipoproteins associated with HDL may act alongside apo AI to inhibit lipid hydroperoxide accumulation. Apolipoprotein E (apo E) appears to display this anti-oxidative activity [423], while apolipoprotein M (apo M) has recently been reported to display anti-oxidative functionality in transgenic mice in addition to facilitating PON1 activity [424, 425]. There is currently little evidence that apolipoprotein J (apoJ) contributes to the anti-oxidative activities of HDL, but it does appear to possess a variety of functions, including endothelial protection.

Myeloperoxidase (MPO)

Cellular systems contributing to oxidative stress *in vivo* include MPO, NADPH oxidase, nitric oxide synthase, and lipoxygenase [394]. It has recently been proposed that MPO and PON1 form a ternary complex with HDL, where the opposing activities of MPO and PON1 determine its oxidation state and whether HDL is pro- or anti-inflammatory / atherogenic [426]. The ratio between these enzyme activities has also been proposed as a marker of HDL functionality and to predict coronary risk [427].

Glutathione Peroxidase (GSPx)

Although, its levels do not appear to affect coronary heart disease risk, GSPx is found associated with HDL, where it has the ability to reduce lipid hydroperoxides [394]. Similarly, trypanosome lytic factor present in higher density HDL also exhibits peroxidase activity and may contribute to the anti-oxidative properties of HDL [394].

Phospholipase A2 (PLA2)

Most PLA2 is associated with LDL, where its activity is an independent risk factor for coronary heart disease [23]. However, there is no evidence that the minor fraction of PLA2 activity on HDL is proatherogenic. Furthermore, PLA2 has overlapping activity with PON1 and it remains unclear just how much of the hydrolysis of platelet activating factor by HDL is due to PLA2 and how much to PON1 [428]. PLA2 on HDL is likely to contribute anti-oxidative activity by the same mechanism as PON1, by hydrolysing lipid hydroperoxides. This activity, which would be pro-atherogenic on LDL in the presence of apo B, may be antiatherogenic in the environment provided by HDL.

Lecithin: Cholesterol Acyl Transferase (LCAT)

Similarly, there is currently limited evidence to support a role for LCAT in the antioxidative activity of HDL [429, 430], but its association with HDL and, like PON1 and PLA2, its role in generating lysophospholipids, does contribute to the hypothesis that HDL provides a safe place to release lysolipids otherwise potentially damaging to cell membranes and other lipoproteins. LCAT esterifies gram-range amounts of cholesterol each day on HDL producing equimolar quantities of lysophosphatidylcholine requiring safe disposal [431].

Cholesteryl Ester Transfer Protein (CETP) and Phospholipid Transfer Protein (PLTP)

The antioxidant activity of HDL occurs following the transfer of lipid hydroperoxides to HDL. The most common lipid to undergo peroxidation is likely to be cholesteryl linoleate with phosphatidyl choline with linoleate in the Sn2 position running close behind. Similar lipids with arachidonate in place of linoleate are also readily susceptible. For any of these to be hydrolyzed on HDL, they must first enter its lipid domains. This process will be assisted by the increased hydrophilicity of, for example the hydroperoxide of cholesteryl linoleate or hydroxyoctadecadienoate [432] which will move it towards the outer more polar region of the LDL particle. Experimentally, no additional facilitator of transfer to HDL is required, but, of course, some cholesteryl ester transfer protein (CETP) and phospholipid transfer proteins (PLTP) are likely to remain in physical association with HDL after its isolation. CETP can accelerate the transfer of both cholesteryl ester and phospholipid hydroperoxides [433]. CETP and/or PLTP may thus be important for the anti-oxidative effect of HDL *in vivo* [433, 434]. Like PON1, PLTP is found predominantly in small, dense HDL, where it is able to interact with apolipoproteins implicated in anti-oxidative function, including apo AI, apo AII, and apo J [394].

Conclusion

The capacity of HDL to protect LDL against oxidative modification is considerable, but its potential therapeutic use to prevent atherosclerosis is as yet unfulfilled. The interaction of lipids with apo AI in HDL provides a lipoprotein particle capable of acquiring potentially toxic lipids and holding them in an environment where they may be safely hydrolysed and from which they may be released to the liver for elimination. PON1, PLA2, and LCAT are present at higher concentrations in small, dense, protein-rich HDL [394], and HDL particles are therefore heterogeneous in their anti-oxidative capacity. PON1 is likely to be critical to the antioxidative capacity of HDL (Figure 5.3), but is likely to require a lipid environment to support its activity. Separation of HDL from PON1 disrupts this and the necessary environment is only imperfectly present with the currently available water-soluble recombinant forms of PON1. Acting together on HDL, PON1, apo AI, apo M, and PLA2 in conjunction with CETP and other lipid transfer proteins probably create a system with both antioxidative and antiglycative properties (see Figure 5.1).

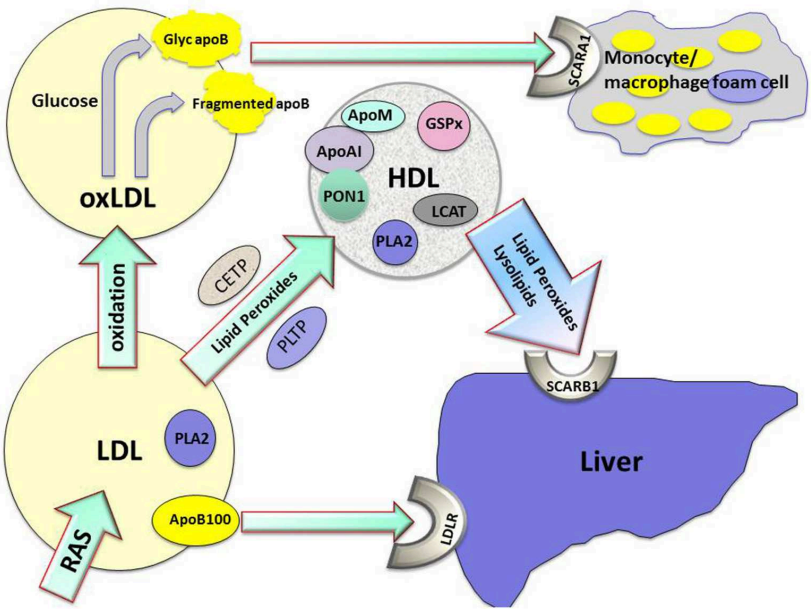

Figure 5.1: The role played by high density lipoprotein (HDL) in the metabolism of lipid hydroperoxides and lysolipids and protection against atherogenesis. *Apo AI, apolipoprotein AI; apoB100, apolipoprotein B100; apoM, apolipoprotein M; CETP, cholesteryl ester transfer protein; glyc apoB, glycated apolipoprotein B; GSPx, glutathione peroxidase; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; oxLDL, oxidised low density lipoprotein; PLA2, phospholipase A2; PLTP, phospholipids transfer protein; PON1, paraoxonase1; RAS, reactive oxygen species; SCARA1, scavenger receptor A1; SCARB1, scavenger receptor B1.*

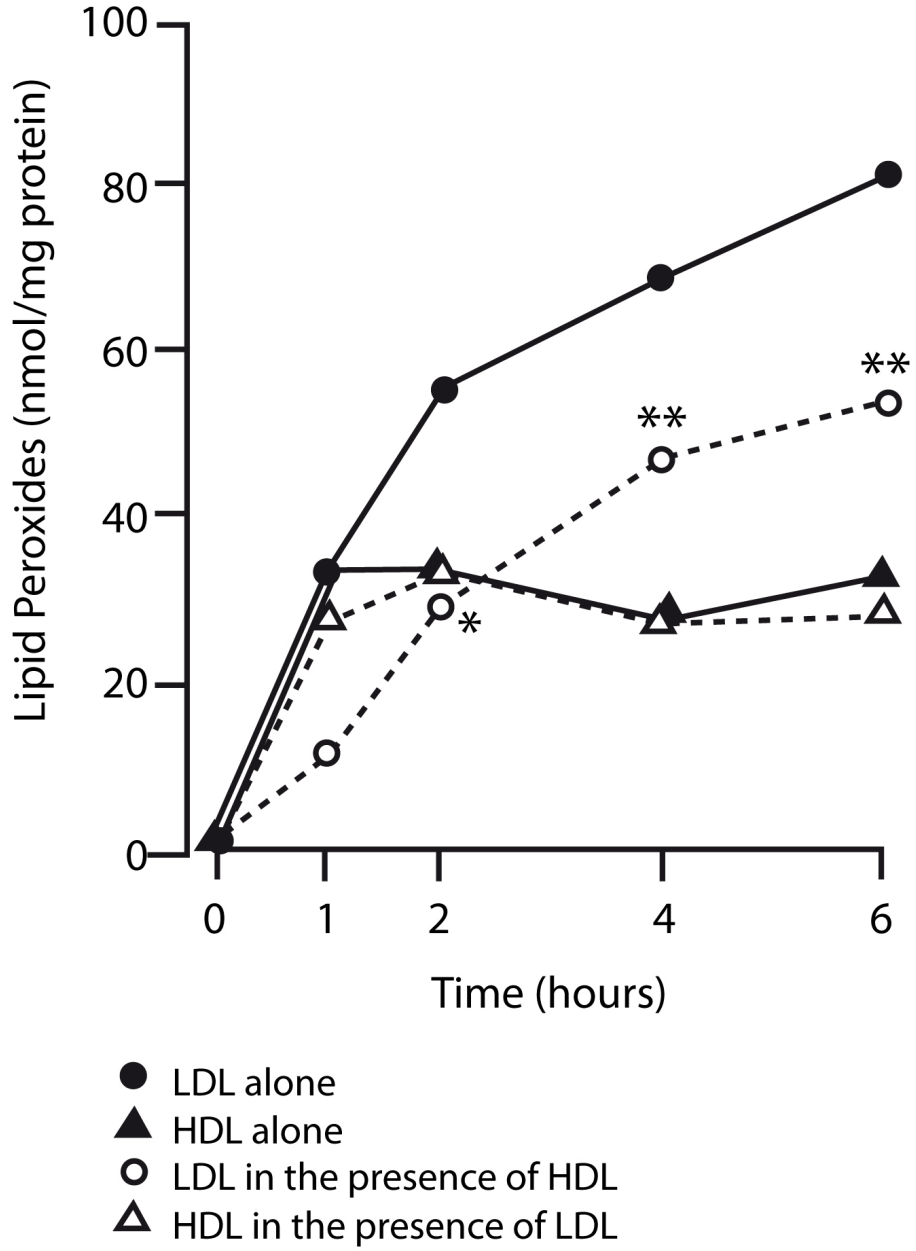

Figure 5.2: Lipid peroxide accumulation on LDL and HDL, incubated under oxidizing conditions (Cu^{2+}) singly and together. *P < 0.05 and **P < 0.001 *vs.* LDL incubated alone

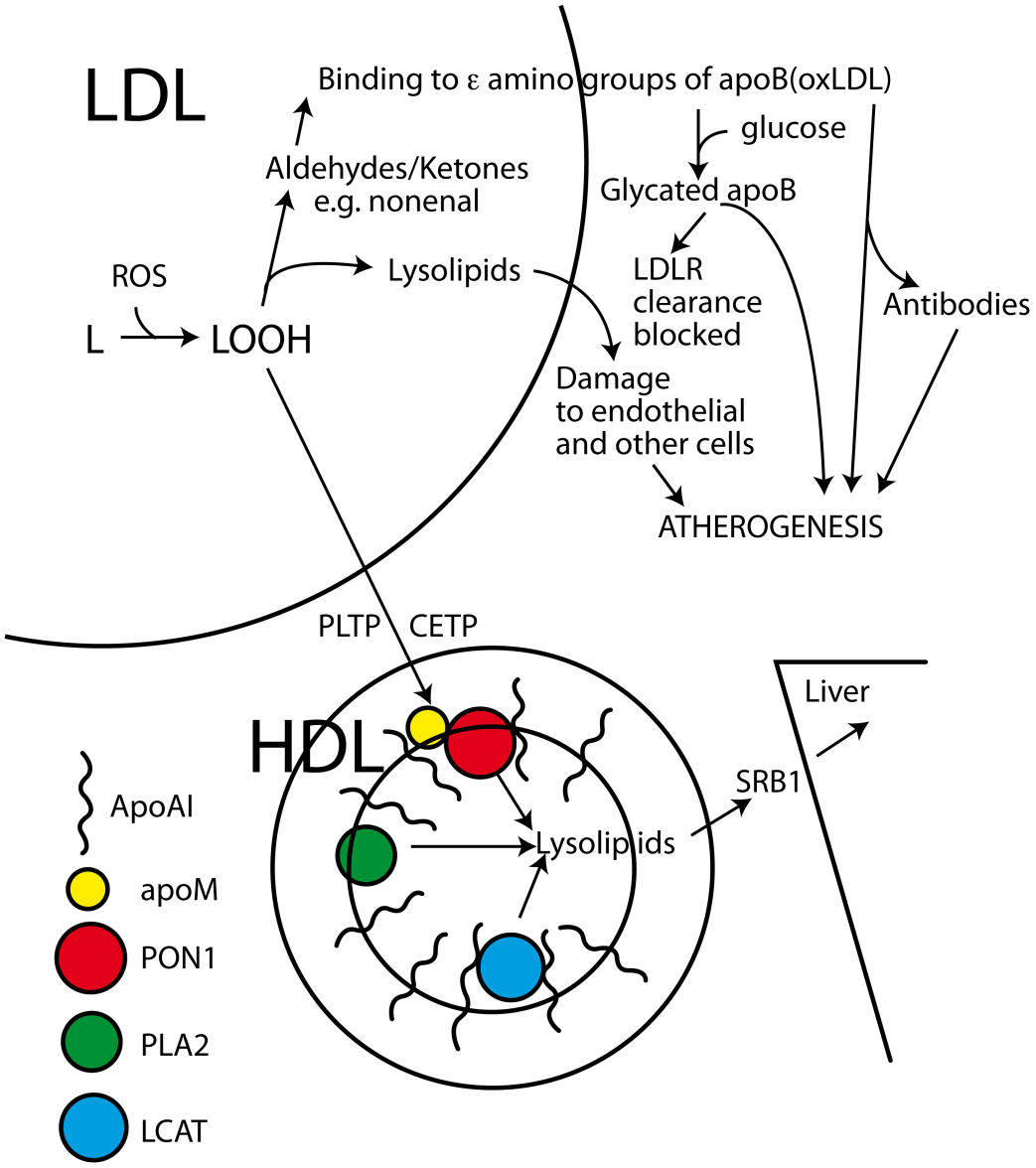

Figure 5.3: Schematic representation of the mechanism by which HDL impedes the atherogenic modification of LDL. *Apo AI: apolipoprotein AI; Apo B: apolipoprotein B; Apo M: apolipoprotein M; CETP: cholesteryl ester transfer protein; LCAT: Lecithin cholesteryl acyl transferase; LDLR: LDL receptor; L: lipid; LOOH: lipid hydroperoxides; Ox-LDL: oxidatively modified LDL; PLA2: phospholipase A2; PLTP: phospholipid transfer protein; PON1: paraoxonase-1; ROS: reactive oxygen species; SRB1: scavenger receptor B1*

How HDL protects against atherogenic modification: paraoxonase 1 and other *dramatis personae*.

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How HDL protects LDL against atherogenic modification: paraoxonase 1 and other *dramatis personae*

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Purpose of review
To summarize the current evidence about how HDL impedes the oxidative and glycative atherogenic modification of LDL.

Recent findings
Paraoxonase 1 (PON1) is located on HDL. Meta-analysis of clinical epidemiological investigations reveals a substantial association of low serum PON1 activity with coronary heart disease incidence independent of other risk factors including HDL cholesterol and apolipoprotein AI (apoAI). Transgenic animal models also indicate an antiatherosclerotic role for PON1. However, highly purified and recombinant PON1 do not retain their antioxidant properties.

Summary
The therapeutic potential of PON1 should be recognized in preventing atherosclerosis and combating infection and organophosphate toxicity. In unleashing this potential, it is important to consider that both highly purified and recombinant PON1 are dissociated from the lipid phase and other components of HDL, such as apoAI and apoM, all of which may be required for HDL (through its PON1 component) to hydrolyze more lipophilic substrates.

Keywords
atherogenesis, glycation, HDL, LDL, lipid peroxidation, paraoxonase

INTRODUCTION

Atherogenesis is complex as is clear from the extraordinarily comprehensive review by Hopkins [1]. Atherologists frequently state that the earliest stage in atheroma is in the arterial endothelium. However, this cannot in itself produce atheroma unless there is also an unnaturally high level of circulating LDL. The fundamental cell type predominating both at the earliest stage of atheroma, the fatty streak, and in the vulnerable shoulder region of the mature, complicated atheromatous lesion is the foam cell. These develop from the engorgement of the cytoplasm of monocyte-macrophages with lipid droplets derived from LDL uptake. In the broth of cytokines created by these cells, smooth muscle cells can migrate from the medial layer of the arterial wall into the lesion developing in the subendothelium where they can also contribute to foam cell formation or transform into fibroblasts producing the collagenous component of atheroma. The adage that there can be no atheroma without cholesterol [2] is true in so many ways. There is no animal model of atheroma in which circulating cholesterol has not been raised usually by diet or genetic manipulation. In nations where the mean cholesterol of the population is less than 5mmol/l, the incidence of premature mortality due to coronary heart disease is low [3,4]. Despite this, in tissue culture, monocyte-macrophages show little inclination in the presence of LDL to become foam cells: LDL receptor

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KEY POINTS

- HDL can protect LDL against oxidative and glycative modifications which permit it to participate in atherogenesis.
- It has been hypothesized that PON1 located on HDL possesses the capacity to hydrolyze lipid hydroperoxides and is largely responsible for the antioxidant effect of HDL.
- Transgenic animal evidence and clinical epidemiology strongly support an antiatherogenic role for PON1.
- Direct in-vitro evidence for the PON1 antioxidant hypothesis has proved controversial, and other HDL components have been proposed to account for the antioxidant capacity of HDL, such as apoAI and apoM. These and other HDL components may interact with PON1 to produce its antioxidant effects. The environment provided for this interaction by HDL may be critical.
- The advent of therapies which increase the PON1-rich portion of HDL while preserving the favourable environment it provides for the antiatherogenic action of PON1 will ultimately unravel the mystery of the antioxidant role of HDL and hopefully prove beneficial.

expression in these cells is too low for this to occur [5]. The discovery that certain chemical modifications of LDL permitted its rapid receptor-mediated uptake by monocyte-macrophages to become foam cells was thus of the greatest importance. It led to the discovery of the scavenger receptors involved in this process [5,6]. It also heralded in the question as to which chemical modification of LDL occurring *in vivo* was responsible for converting native LDL to a ligand for these receptors. Oxidation, glycation and homocysteinylation have been front-runners with oxidation being the punters' favourite in terms of research activity. However, it did not prove a winner when chain-breaking antioxidants underwent clinical trials [7], and we must look again both at the nature of the potentially atherogenic LDL modifications that can occur biologically and the mechanisms by which they arise and the systems which can oppose these, in which HDL appears to have a major role. This review will focus on the latest developments in the latter.

It has been proposed that the HDL component that impedes oxidative modification of LDL is paraoxonase 1 (PON1) [8–11], an enzyme located almost exclusively on HDL, initially studied because of its capacity to hydrolyze organophosphate pesticides and nerve gasses. Recently, we have concentrated on glycation of LDL as a more persistent and potentially important atherogenic modification of LDL [12] as opposed to simple oxidation for which there are numerous metabolic safeguards. This has led us back to PON1 [13]. Although there are strands of evidence strongly supportive of our hypothesis that it is PON1 located on HDL that impedes atherogenic modifications of LDL, there are a number of observations that are not easily compatible with it. It is often examination of apparent inconsistencies in evidence that leads to new discoveries, which is a major justification for another article on this subject. The other HDL components that have been conjectured to be responsible will also be reviewed. The evidence for these emerges as probably less convincing than for PON1, although they may by coexisting in the same location provide a coordinated whole which provides a safe environment to receive lipid-derived noxious substances from not only other lipoproteins, such as LDL, but also from cell membranes during the passage of HDL through the tissue fluid, where it is the dominant lipoprotein species, and transporting them to the liver for elimination. Solving the central mysteries of the protective mechanisms, which have evolved aboard the HDL particle, could reveal important pathways, which if manipulated, could prevent atherosclerosis.

ATHEROGENIC LDL MODIFICATION

What makes LDL a ligand for macrophage scavenger receptors and less readily able to bind to the physiological LDL receptor? Following the discovery that this could be achieved by chemical modifications of LDL, such as acetylation, the search was on for naturally occurring modifications with a similar effect [5]. Dan Steinberg's group [5] advanced the hypothesis that lipid peroxidation products produced on LDL when it was subjected to attack by oxygen-free radicals were responsible for the changes in the apolipoprotein B of LDL which altered its receptor binding preferences [6]. This was both profound and compelling at the time and remains so. The disappointing lack of effect of chain-breaking antioxidants [7], although a setback for the hypothesis, can perhaps be explained because once they have been oxidized in preference to say unsaturated lipids, they themselves become pro-oxidant. Furthermore, it seems unlikely that the human organism with its huge demand for oxidative metabolism should be vulnerable to the lack of pharmacological quantities of fat-soluble antioxidant vitamins. Perhaps unsurprisingly, given the safeguards against oxygen-free radicals damaging tissues [6], only low circulating concentrations of, for example oxidized LDL, are detectable, although it has been argued that higher levels might occur in sites where LDL is sequestered and perhaps this

might include the arterial wall [6]. It has also been argued that perhaps antibodies to oxidatively modified LDL are involved [5] Nevertheless, it would be logical to consider that other naturally occurring modifications of LDL with similar receptor binding preferences to oxidatively modified LDL (oxLDL), but present more ubiquitously and at higher concentration, might be critical to atherogenesis. Glycated LDL (glycLDL) springs to attention for such a role. Many years ago, we were surprised to discover using boron affinity chromatography and a sensitive apoB assay that glycated apoB was present in the circulation of apparently healthy people at around 2–3 mg/dl [14]. This level could double in diabetes and in nondiabetic patients with high levels of LDL, such as those with familial hypercholesterolaemia [14]. This has been confirmed by immunoassays detecting epitopes unique to glycLDL, and it has been found that in-vivo small dense LDL is more heavily glycated than other LDL subfractions and is more susceptible to in-vitro glycation [15–17]. GlycLDL has for many years been known to be removed more slowly than unmodified LDL by route(s) not involving the LDL receptor [18]. Its circulating concentration is decreased in people receiving statin treatment [16].

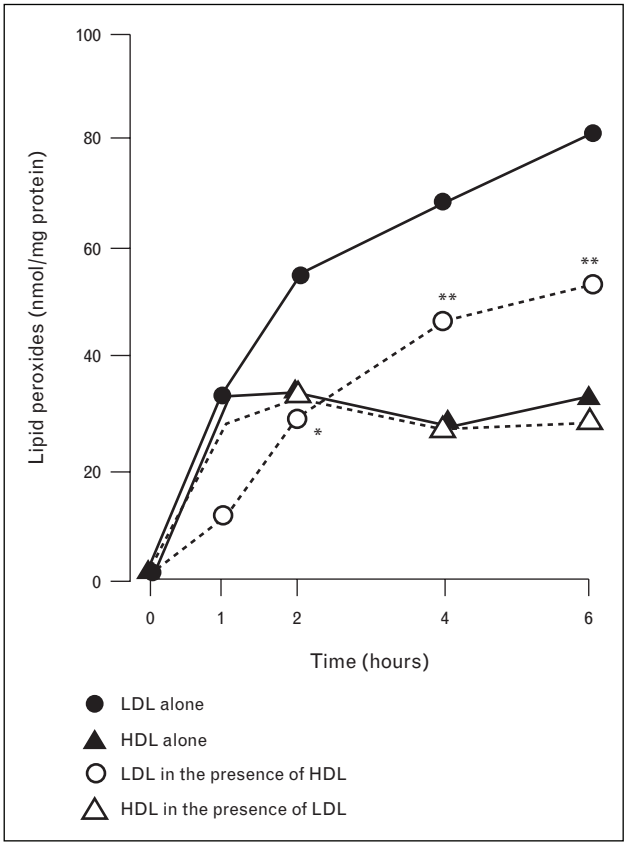

FIGURE 1. Lipid peroxide accumulation on LDL and HDL, incubated under oxidizing conditions (Cu^{2+}) singly and together [11]. $^{*}P < 0.05$ and $^{**}P < 0.001$ versus LDL incubated alone.

ANTIOXIDATIVE ROLE OF HDL

Workers in St Petersburg [19] and San Diego [20] showed that lipid hydroperoxides could be transferred from LDL to HDL. Lipid hydroperoxides formed on LDL, as a result of their greater hydrophilicity, would be expected to migrate to its surface, facilitating the transfer to HDL. It was speculated that HDL might thus provide a pathway for the passage of lipid peroxides to the liver via hepatic scavenger receptors. Although this mechanism undoubtedly exists, particularly providing a means of safely disposing of lysophospholipids from HDL to the liver (see later discussion), it was not realized until 1991 that HDL metabolizes lipid hydroperoxides preventing their accumulation both on it and on LDL and thus impeding the structural modification of LDL by the products of lipid peroxidation [8]. When HDL was incubated with LDL under oxidizing conditions, the accumulation of lipid peroxides on LDL was decreased, but the concentration of lipid peroxides on HDL remained similar to that observed when HDL alone underwent oxidation (Fig. 1) [11]. The lipid peroxides accumulating on HDL, regardless of whether LDL is present, rapidly reach a plateau. When LDL was oxidized in the presence of HDL, fewer lipid peroxides in the system as a whole were present than when LDL alone was oxidized [8–10]. This effect of HDL is clearly present within 3 h by which time typically more than 50% of the lipid peroxidation of LDL, which would occur under the same oxidizing conditions if HDL were not present, has been prevented. The effect, which persists for several more hours, is not due to chain-breaking antioxidants or transition metal chelation, but due to enzymatic activity present on HDL [9–11]. It should also be borne in mind that these effects of HDL are observed *in vitro* with similar protein concentrations of LDL and HDL. When HDL concentrations exceed those of LDL as they do in the tissue fluid, even greater suppression of LDL oxidation is to be expected. The antioxidant effects of HDL have since been demonstrated by others in a variety of experimental systems and have proved relatively uncontroversial [21,22]. We further concluded that a major component of this antioxidative activity was due to PON1. However, this, as we discuss later, has been more contentious, but it is important to emphasize that the evidence that PON1 is antiatherogenic is less contested: it is whether it does so by impeding lipid oxidation that is argued.

ANTIGLYCATIVE ACTIVITY OF HDL

Experimentally, HDL can impede the glycative modification of LDL, and this property of HDL is more marked with HDL obtained from people with higher serum PON1 activity [13]. In these experiments, to avoid lipid peroxidation as a confounding variable, external oxygen was eliminated as a source of free radicals. However, it was noted that LDL is under these conditions relatively resistant to glycation and that even with very high concentration of glucose it is not possible to glycate LDL to the extent found in many people with diabetes [12]. A small amount of oxidation does accompany in-vitro glycation, and the process is best regarded as glycoxidation [17]. Lipid peroxidation of LDL accompanying in-vitro glycation is impeded in the presence of HDL, and it is possible that adduction of lipid peroxidation products to the ε amino groups of lysine residues of apoB *in vivo* renders these groups more susceptible to combination with glucose. In-vivo oxygen-free radical exposure of LDL may predispose to glycation and explain the high levels of circulating glycLDL. HDL by impeding LDL oxidation may thus in turn slow its glycation. An alternative hypothesis is that gluconolactone is the active metabolite of glucose important for apoB glycation [23] and perhaps the lactonase activity of PON1 impedes it.

PARAOXONASE 1

PON1 is produced in the liver and circulates on HDL. It possesses antiatherosclerotic properties and the capacity to hydrolyze lipid hydroperoxides, and is one of the cast of players responsible for the antioxidant effect of HDL.

Direct experimental evidence

We partially purified HDL components and found that the PON1-containing fraction was most active in impeding Cu^{2+}-induced lipid peroxide accumulation on LDL [10,24]. Since then, several arguments have been advanced that it is not PON1, which is responsible. Firstly, because PON1 is dependent on Ca^{2+} for its activity, the use of phosphate buffers in our work was criticized [25]. There is, however, variation in the Ca^{2+} concentration necessary for the hydrolysis of different substrates by PON1. Paraoxon hydrolysis commonly used to detect PON1 activity is highly influenced by the Ca^{2+} concentration [26], but lipid hydroperoxide hydrolysis may be less so and persist in the phosphate buffers enriched with Ca^{2+} used in our experiments. Secondly, it was reported that both highly purified preparations of PON1 isolated from HDL and recombinant water-soluble variants of PON1 do not hydrolyze lipid peroxides [22,25,27]. However, it is exceptionally difficult to separate PON1 from other HDL components, such as apolipoprotein AI (apoAI) and phospholipase A2 (PLA2), without subjecting it to conditions in which it becomes dissociated from the lipid environment essential for its catalytic activity, such as active site floppiness [28], in reactions involving lipid substrates. In the case of recombinant PON1, the creation of mutations making it more polar [27,29▪] is similarly going to compromise its activity in hydrolyzing hydrophobic substrates [26]. The trade-off between ease of isolation, which has proved difficult for the more lipophilic mutants, against loss of function in those that are more water-soluble will be critically important in the development of recombinant PON1 for therapeutic use, a subject recently reviewed [29▪]. The direct biochemical approach to determine the contribution of PON1 to the antioxidative activity of HDL clearly has its limitations, and evidence from other sources must be considered.

Epidemiological evidence linking paraoxonase 1 and atherosclerosis

The PON1 concentration measured within 2 h of the onset of symptoms of myocardial infarction was found to be low [30]. Following the initial report from a prospective epidemiological study that PON1 activity was independently inversely associated with coronary events [31], numerous other prospective investigations appeared, the results of which have recently been subjected to a meta-analysis, which considered 47 such studies involving 9853 coronary heart disease cases and 11 408 controls [32]. PON1 activity was 19% lower in the cases ($P < 10^{-5}$). The largest single cohort study to date (Cleveland Clinic GeneBank study) involving 3668 patients following coronary angiography revealed a greater than two-fold risk of new cardiovascular events in the lowest compared with the highest quartile of serum PON1 measured as aryl esterase activity [33]. For the plausibility of the concept that PON1 is antioxidant, some reviewers [34▪] have emphasized studies in which circulating levels of lipid peroxidation products as well as coronary incidents were linked to PON1 activity, such as that by Bhattacharyya *et al.* [35].

In terms of Mendelian epidemiology, there have been many case–control studies of PON1 genotypes influencing PON1 activity [36,37]. The most numerous of these are of the R192Q genotype. HDL from people who are 192QQ homozygotes is most effective in preventing the accumulation of lipid peroxides on LDL [38]. In two meta-analyses, relative

cardiovascular disease (CVD) risk was increased in people possessing an 192R allele as opposed to QQ homozygotes [36,37]. In both of these, there was evidence of publication bias in favour of trials with a positive outcome and in both a more marked effect was evident in diabetes. The conclusion of the authors of one of the meta-analyses was that it did not exclude the possibility of an effect of the 192 polymorphism on CVD risk [36], whereas the other meta-analysis was interpreted as denying such an effect [37]. This latter conclusion was, however, based on the erroneous assumption that a large effect of the 192 polymorphism on CVD risk was to be expected. Although the effect of the 192 polymorphism on PON1 activity measured using paraoxon as substrate is large, it is nonexistent with other substrates, such as phenyl acetate, and, in the case of lipid peroxides is reversed with the R allele having the higher catalytic activity towards paraoxon hydrolysis [11]. That serum PON1 activity (measured as paraoxon or phenyl acetate hydrolysis), representing the integral of genetic and acquired influences, is a stronger determinant of coronary risk than genotype is, however, undeniable and amply confirmed by the recent findings of the Cleveland Clinic GeneBank study [33]. There is, however, stronger evidence that genetic polymorphisms determine individual susceptibility to organophosphate poisoning [39–41]. This is likely to represent different specificities for different substrates with the nature of the PON1 alloenzyme having more influence when the substrate is hydrolyzed at relatively low rates as it is, for example with paraoxon, and none when reaction rates are high as with phenyl acetate as substrate. Hydrolysis of the putative PON1 substrate which is the basis of the antiatherogenic action of PON1 is either unaffected by the particular alloenzyme expressed or the effect is too small to detect in an epidemiological investigation when other genetic and acquired factors are influencing overall PON1 concentration and specific activity. Research into the association of genetic polymorphism of PON1 has not proved entirely negative outside the field of toxicology, because the earlier finding that diabetic retinopathy is related to the L55 M polymorphism of PON1 [42], for which there remains no explanation, has emerged strongly from meta-analysis [43].

Genetic evidence linking paraoxonase 1 and atherosclerosis

The HDL from avian species which lack serum PON activity has no capacity to protect human LDL against lipid peroxidation [44]. Ablation of mouse *PON1* not only renders mice more susceptible to organophosphate poisoning, but also increases their susceptibility to atherosclerosis and compromises the capacity of their HDL to prevent the accumulation of lipid peroxides on human LDL [45–47]. On the other hand, transgenic rodent models expressing human *PON1* are protected against atherosclerosis [48–50]. In both the knockout and transgenic animals, these effects are achieved without any major change in lipoprotein metabolism. In contrast, knockout and transgenic animal models of *APOAI, lecithin: cholesterol acyl transferase (LCAT) a*nd *PLA2* have little discernible effect on atherosclerosis susceptibility, despite often substantial changes in lipoprotein metabolism [51–55]. For example, some five copies of the human *APOAI* gene must be expressed in rabbits to prevent atherosclerosis [51], whereas expression of two copies is ineffective even though it almost doubles HDL cholesterol concentration [53].

Factors affecting serum paraoxonase 1 activity

We and others have reviewed these previously [56,57], and it is noteworthy how many medical conditions linked with decreased serum PON1 activity are associated with increased CVD risk. These include type 1 and 2 diabetes mellitus, chronic renal disease, familial hypercholesterolaemia and inflammatory disorders, such as rheumatoid arthritis and systemic lupus erythematosus.

PON1 activity is decreased in both type 1 and 2 diabetes [42,58], and lower levels are associated with microvascular complications [42,58–62]. *PON1* and *PON2* genes have also been linked with diabetic microvascular complications [61,63–65] and with susceptibility to diabetes and severity of glycaemia [42,66,67]. Often, it has been conjectured that the association is because oxidation may be involved in pancreatic β cell dysfunction or in the genesis of microvascular disease. However, there is evidence that HDL rich in PON1 can impede the in-vitro glycation of the apoB of LDL [13]. LDL glycation is a potentially atherogenic modification of LDL, and glycation of other proteins may be critical for the development of microvascular disease. The prospect that HDL and PON enzymes may be important in prevention of post-translational protein glycation is thus intriguing [68].

Paraoxonase 1-raising therapy

There is evidence that the parenteral administration of partially purified PON1 can ameliorate experimental atherosclerosis and a patent for PON1 as an antiatherogenic agent in humans exists [69].

Recombinant PON1 could be used for this purpose too if its atherosclerotic properties were retained as has been earlier discussed [27,29▪,70]. Intraperitoneal injection of recombinant PON1 in mice increased HDL aryl esterase and lactonase activities, the resistance of HDL to oxidation and HDL-induced cholesterol efflux and decreased macrophage-mediated LDL oxidation [71]. Much of the current impetus for recombinant PON1 therapy is, however, on the one hand to protect people likely to be exposed to organophosphates or as an antidote in those who have been exposed [29▪,72] and on the other hand, to combat serious infections [34▪]. Another development has been apoAI-mimetic peptides, some of which are orally active [73]. It might be thought that these mimic simply the actions of apoAI, but they create circulating lipid complexes which are a magnet for other HDL components such as PON1 [74]. We know that HDL is drawn to atheromatous lesions, where immunolocalization of both apoAI and PON1 occurs [75].

APOLIPOPROTEIN AI

ApoAI is a strong contender as a major component of the antioxidant effect of HDL. Indeed, it would be difficult to conceive otherwise, because with its extraordinary detergent properties it is essential for the very structure of HDL and the maintenance of the lipid environment in which enzymes such as PON1 and LCAT can operate [76▪]. It is thus an essential cofactor for both. It also creates a safe environment for the release of lysolipids, such as lysophosphatidylcholine in the case of LCAT and its subsequent transfer to the liver. Despite experimental evidence that lipid-protein complexes containing only apoA1 can protect LDL against oxidation, which has been extensively reviewed [34▪], neither human genetic disorders nor animal genetic models provide an altogether convincing case that apoAI is directly antiatherogenic unless marked increases in HDL occur comparable to those achieved by infusion therapy and then, of course, many other components of HDL will be increased including phospholipids and other proteins, such as PON1.

APOLIPOPROTEIN AII

Apolipoprotein AII (ApoAII)-containing HDL particles tend to be larger and possess less antioxidant activity than smaller ones with higher apoAI content [34▪]. Both human studies and evidence from transgenic animal models suggest that apoAII suppresses PON1 binding to HDL [77]. Mice expressing human apoAII and apoAI are more susceptible to atherosclerosis than those expressing only apoAI. Delivery of the antioxidant capacity of HDL to vulnerable tissue sites may be fundamental to its function, and smaller HDL particles are likely to predominate in the tissue fluid for the same reason that HDL predominates over LDL: the smaller the lipoprotein, the higher its rate of ultrafiltration. PON1 activity is present in tissue fluid [78].

APOLIPOPROTEIN M

Recently, this has been reported to act as a platform for PON1 bringing it together with its substrates [79▪▪,80]. It is likely to be a cofactor with apoAI for PON1 reactions.

APOLIPOPROTEIN J

Apolipoprotein J (Apo J) is a component of HDL which may serve as a circulating reservoir of this protein which has a variety of possible functions including implication in protecting endothelial and other cell membranes against disruption. As such, it contributes to the emerging concept that HDL is the repository of factors protective against tissue damage, but there is currently little evidence that it contributes to the antioxidative property of HDL [81].

LECITHIN: CHOLESTEROL ACYL TRANSFERASE

Evidence that LCAT is involved in the antioxidative function of HDL is limited [82,83], but its colocation on HDL with other circulating enzymatic activities, such as PON1 and PLA2, catalyzing reactions also generating lysolipids is hardly likely to be coincidence and suggests that HDL is a safe place to produce such substances potentially damaging to cell membranes and other lipoproteins. LCAT esterifies gram-range amounts of cholesterol each day on HDL producing equimolar quantities of lysophosphatidylcholine requiring safe disposal [84].

PHOSPHOLIPASE A2

PLA2 or platelet-activating factor is largely present on LDL, and its concentration reflects that of LDL [85]. Its circulating activity is a risk factor for coronary heart disease. However, there is no evidence that the minor fraction of PLA2 activity on HDL is proatherogenic. Furthermore, as it has overlapping activity with PON1 and the two activities (or enzymes) are difficult to separate, it is unclear just how much of the hydrolysis of platelet-activating factor by HDL is due to PLA2 and how much to PON1 [86]. Probably, any true PLA2 activity on HDL, like that of PON1, contributes to its antioxidant

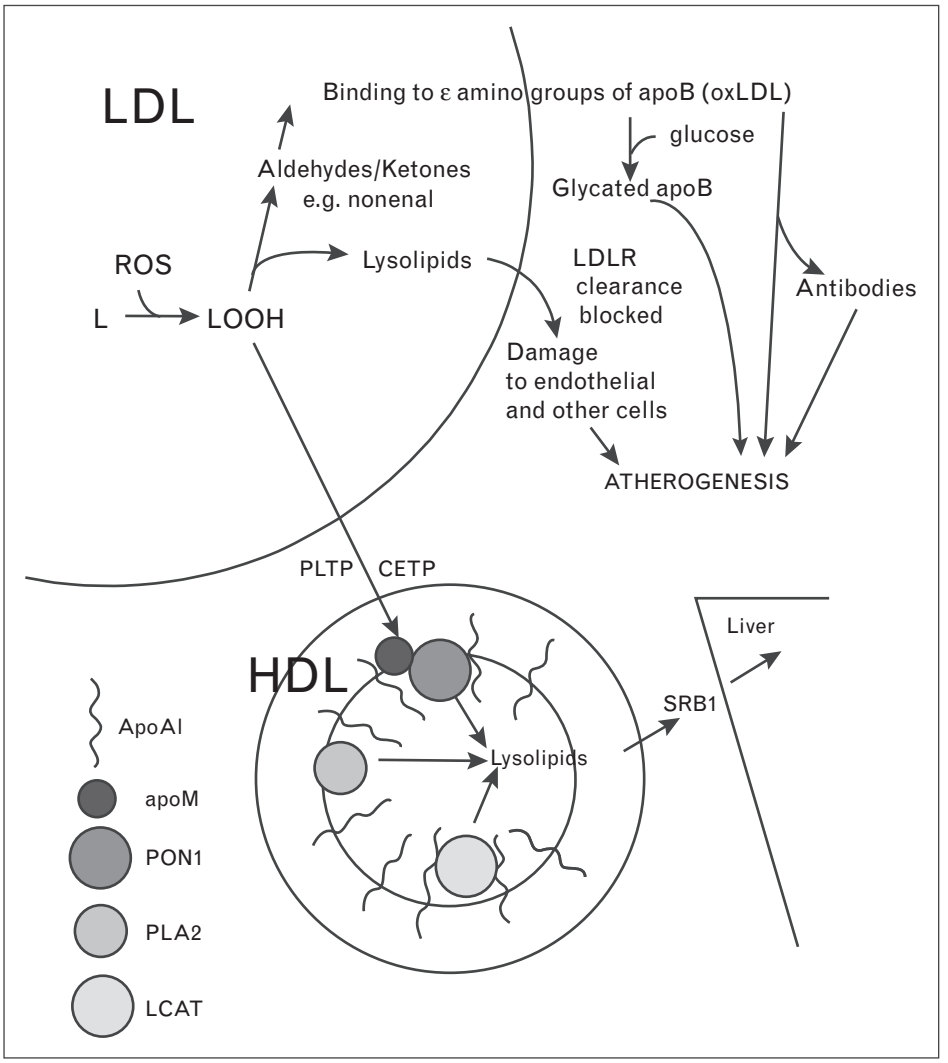

FIGURE 2. Schematic representation of the mechanism by which HDL impedes the atherogenic modification of LDL. ApoAI, apolipoprotein AI; ApoB, apolipoprotein B; ApoM, apolipoprotein M; CETP, cholesteryl ester transfer protein; LCAT, lecithin; cholesterol acyl transferase; LDLR, LDL receptor; L, lipid; LOOH, lipid hydroperoxide; Ox-LDL, oxidatively modified LDL; PLA2, phospholipase A2; PLTP, phospholipid transfer protein; PON1, paraoxonase 1; ROS, reactive oxygen species; SRB1, scavenger receptor B1.

effect perhaps by hydrolyzing lipid hydroperoxides. A similar activity on LDL, because it can yield toxic products in an unsafe environment, because of the presence of apoB, would explain its proatherogenicity there.

OTHER PARAOXONASES

PON1 has two other family members, *PON2* and *PON3*. *PON2* is almost exclusively expressed intracellularly, whereas human HDL does contain PON3, albeit in lesser quantities than PON1. The hydrolytic activity of PON3 may be primarily as a lactonase [27] similar to PON1. *PON3* ablated mice are atherosclerosis prone [87], but the reason for its evolutionary conservation is currently unclear.

MYELOPEROXIDASE

It has recently been proposed that HDL provides a scaffold for the opposing activities of myeloperoxidase and PON1 determining its oxidation state and whether it is proinflammatory or anti-inflammatory/atherogenic [88]. Another study suggests the ratio between these two enzyme activities may predict coronary risk [89].

CHOLESTERYL ESTER TRANSFER PROTEIN AND PHOSPHOLIPID TRANSFER PROTEIN

The antioxidant activity located on HDL is not envisaged to protect LDL by, itself, transferring to it. Rather, it is considered to occur after transfer of

lipid hydroperoxides to HDL. The most common lipid to undergo peroxidation is likely to be cholesteryl linoleate with phosphatidyl choline with linoleate in the Sn2 position running close behind. Similar lipids with arachidonate in place of linoleate are also readily susceptible. For any of these to be hydrolyzed on HDL, they must first enter its lipid domains. This process will be assisted by the increased hydrophilicity of, for example the hydroperoxide of cholesteryl linoleate or hydroxyoctadecadienoate [90] which will move it towards the outer more polar region of the LDL particle. Experimentally, no additional facilitator of transfer to HDL is required, but, of course, some cholesteryl ester transfer protein (CETP) and phospholipid transfer proteins (PLTP) are likely to remain in physical association with HDL after its ultracentrifugal isolation and thus to be present. In-vivo CETP and/or PLTP may be important for the antioxidative effect of HDL [91,92].

CONCLUSION

The capacity of HDL to protect LDL against oxidative modification is considerable and, because of its potential to prevent atherosclerosis, further exploration is attractive. The interaction of lipids with apoAI in HDL is undoubtedly an important element, as it provides a lipoprotein particle capable of acquiring potentially toxic lipids and apprehending them in an environment where they may be safely hydrolyzed and from which they may be released to the liver for elimination. Another major player is likely to be PON1 (Fig. 2). It is likely that this unusual enzyme requires a lipid environment for its antioxidative activity. This is irreversibly lost when PON1 is rigorously separated from HDL and is only weakly present in recombinant forms of PON1, which have thus far obtained only as more water-soluble mutated versions. This has led some to question whether PON1 is critical to the antioxidative function of HDL. However, evidence is from rodent models in which *PON1* has been ablated or overexpressed, from epidemiology and from disorders associated with precocious atherosclerosis are persuasive that PON1 is antiatherogenic. Thus, to deny its antioxidative activity is to entertain the proposition that its antiatherosclerotic role has been serendipitously discovered and has some as yet, unknown explanation. PON1 displays astounding substrate promiscuity. Polymorphisms, such as Q192R, important in determining individual susceptibility to organophosphates, probably have little effect in impeding lipid peroxidation and none at all against rapidly hydrolyzed substrates, such as phenyl acetate. There has been considerable interest in the recent finding that PON1 has high lactonase activity which contributes to innate immunity, and it has been proposed that this may have led to its evolutionary conservation. The concept should not, however, be dismissed that PON1 along with other *dramatis personae* located on HDL (ApoAI, ApoM, ApoJ, CETP and other lipid transfer proteins, LCAT, PLA2 and other PONs) play roles in creating a lipoprotein which protects and restores outer cell membranes that are bathed in HDL, which is the most abundant lipoprotein in tissue fluid. To its antioxidant function can probably now be added an antiglycative action of HDL. The outer surface of LDL is similar to a cell membrane, and it would be expected that it too would enjoy this protection against oxidative and glycative damage otherwise injurious in promoting atherogenesis.

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Conflicts of interest

There are no conflicts of interest.

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Antioxidant properties of HDL

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High-density lipoprotein (HDL) provides a pathway for the passage of lipid peroxides and lysophospholipids to the liver via hepatic scavenger receptors. Perhaps more importantly, HDL actually metabolizes lipid hydroperoxides preventing their accumulation on low-density lipoprotein (LDL), thus impeding its atherogenic structural modification. A number of candidates have been suggested to be responsible for HDL's antioxidant function, with paraoxonase-1 (PON1) perhaps the most prominent. Here we review the evidence for HDL anti-oxidative function and the potential contributions of apolipoproteins, lipid transfer proteins, paraoxonases and other enzymes associated with HDL.

Keywords: apolipoprotein A1, glycated low-density lipoprotein, high-density lipoprotein, oxidized low-density lipoprotein, paraoxonase 1

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Introduction

Whilst atherogenesis is a complex process, macrophage-derived foam cell formation resulting from the uptake of circulating low-density lipoprotein (LDL) is of fundamental importance. Despite this, foam cell formation with LDL is impossible to instigate *in vitro* due to insufficient monocyte-macrophage LDL receptor expression (Steinberg and Witztum, 2010). It was, however, discovered that experimental chemical modification of LDL permitted its rapid receptor-mediated uptake by monocyte-macrophages to form foam cells. This led to the identification of the scavenger receptors (Stocker and Keaney, 2004; Steinberg and Witztum, 2010) and opened new avenues of research to identify possible *in vivo* atherogenic modifications of LDL. Oxidation, glycation, and homocysteinylation have all been explored. Although, clinical trials of chain-breaking antioxidants proved disappointing in the prevention of atherosclerotic cardiovascular disease (Heart Protection Study Collaborative, 2002), other systems which might oppose potentially atherogenic LDL modifications, including high-density lipoprotein (HDL) merit further attention.

We have contributed to the notion that paraoxonase 1 (PON1), an enzyme located almost exclusively on HDL, is important in impeding oxidative modification of LDL (Mackness et al., 1991, 1993; Durrington et al., 2001). Our recent work has focused on glycation as an atherogenic modification of LDL and this too has led us back to PON1 (Younis et al., 2013). Other HDL components have also been conjectured to be involved in preventing atherogenic LDL modification and evidence increasingly points to a coordination of these with PON1. Understanding these protective mechanisms might reveal important pathways which could be manipulated therapeutically to prevent atherosclerosis.

Atherogenic LDL Modification

The discovery that chemical modification of LDL by acetylation increases its affinity for macrophage scavenger receptors and reduces binding to the physiological LDL receptor led to

a search for naturally occurring modifications which might have similar effects (Steinberg and Witztum, 2010). The ensuing hypothesis that lipid peroxidation products produced on LDL when subjected to attack by oxygen free radicals are responsible for changes in the apolipoprotein B100 (apoB) of LDL which alter its receptor binding preferences remains compelling despite the disappointing lack of effect of chain-breaking antioxidants (Heart Protection Study Collaborative, 2002; Stocker and Keaney, 2004; Steinberg and Witztum, 2010). Perhaps unsurprisingly given the safeguards against oxygen free radical damage, oxidized LDL is only found at low circulating concentrations, although it has been argued that higher levels might occur at sites where LDL is sequestered and this might include the arterial wall (Stocker and Keaney, 2004).

We were surprised to discover that glycated apoB was present in the circulation at relatively high concentrations of around 2–3 mg/dl in healthy people and at higher levels in diabetes and in hypercholesterolaemia (Tames et al., 1992). This has been confirmed by immunoassays detecting epitopes unique to glycated LDL and we have also shown that atherogenic small dense LDL is more heavily glycated than other LDL subfractions *in vivo*, and is more susceptible to glycation *in vitro* (Younis et al., 2009, 2010). Glycated LDL also has a longer circulating half-life than unmodified LDL, and is removed from the circulation by route(s) not involving the LDL receptor. Interestingly, statins also reduce circulating concentrations, likely by reducing LDL available to undergo glycation (Younis et al., 2010).

Homocysteine may also be atherogenic; thiolation of LDL free amino groups by homocysteine thiolactone increases its uptake by macrophages (McCully, 1993). Interestingly the lactonase activity of PON1 will detoxify homocysteine thiolactone in addition to its role in preventing LDL oxidation (see later).

HDL Antioxidative Activity

Lipid hydroperoxides formed on LDL will migrate to its surface as a result of their greater hydrophilicity, facilitating their transfer to HDL (Parthasarathy et al., 1990). This transfer can occur directly between lipoprotein phospholipid monolayers, but may be assisted by lipid transfer proteins (see **Figure 1**). HDL might thus provide a pathway for the passage of lipid peroxides and lysophospholipids to the liver via hepatic scavenger receptors. Perhaps more importantly, HDL actually metabolizes lipid hydroperoxides preventing their accumulation, consequently impeding the atherogenic structural modification of LDL (Mackness et al., 1991). We have observed that when HDL is incubated with LDL under oxidizing conditions the accumulation of lipid peroxides on LDL is decreased, but the concentration of lipid peroxides on HDL remains similar to that observed when HDL alone is oxidized (Mackness et al., 1991, 1993). This effect of HDL is obvious within 3 h, by which time typically more than 50% of the lipid peroxidation of LDL which would occur in the absence of HDL has been prevented. These results suggest that this effect is related to enzymatic activity associated with HDL, and not chain-breaking antioxidants or transition metal chelation (Mackness et al., 1993; Durrington et al., 2001). It should also be noted that this anti-oxidative function of HDL is observed *in vitro* with similar protein concentrations of LDL and HDL; greater suppression of LDL oxidation might be expected when HDL concentrations exceed those of LDL as they do in the interstitial fluid. In fact, the accumulation of oxidized lipids in HDL likely results not only from their transfer from LDL but also from triglyceride-rich remnant particles and endothelial cells. The antioxidant effects of HDL are now well established and have been demonstrated in a number of experimental systems (Kontush and Chapman, 2010).

HDL Antiglycative Activity

We have also shown that HDL can impede the modification of LDL by glycation *in vitro*, and that this anti-glycative function of HDL is more marked with HDL obtained from people with higher serum PON1 activity (Younis et al., 2013). We noted in these experiments that LDL is relatively resistant to glycation in the absence of oxygen, such that supraphysiological glucose concentrations are required. Oxidation appears to accompany *in vitro* glycation and the process is best regarded as glycoxidation. The lipid peroxidation of LDL that accompanies *in vitro* glycation is also impeded in the presence of HDL. Adduction of lipid peroxidation products to the ε amino groups of apoB lysine residues *in vivo* may render these groups more susceptible to combination with glucose. Thus, *in vivo* exposure of LDL to oxygen free radicals may predispose to glycation and explain the observed high levels of circulating glycated LDL. The effect of HDL on glycation may thus be related to its anti-oxidative function. An alternative hypothesis is that the oxidized analog of glucose, gluconolactone, is more involved in apoB glycation, and that this step might be affected by PON1's lactonase activity.

Paraoxonase 1

PON1 is produced in the liver and circulates on HDL. There is a significant body of evidence to support a role for PON1 in atherosclerosis, and in particular against oxidation, not least its capacity to hydrolyze lipid hydroperoxides.

We demonstrated that the HDL fraction containing PON1 was most active in impeding Cu^{2+} induced lipid peroxide accumulation on LDL (Mackness et al., 1993). It has since been suggested that it is not PON1 which is responsible for this effect, an argument supported by reports that more highly purified PON1 isolated from HDL and recombinant water-soluble variants of PON1 do not hydrolyze lipid peroxides (Draganov et al., 2005; Kontush and Chapman, 2010). It is however exceptionally difficult to separate PON1 from other HDL components, such as apolipoprotein AI (apoAI) and phospholipase A2 (PLA2), without subjecting it to conditions which might affect its catalytic activity (Ben-David et al., 2015). Similarly, the increased polarity of recombinant PON1 would be expected to compromise its ability to hydrolyze hydrophobic substrates (Harel et al., 2004; Draganov et al., 2005; Bajaj et al., 2014). More lipophilic recombinant PON1 might be expected to have improved functionality, but is more difficult to isolate, a factor which will prove important in the development of

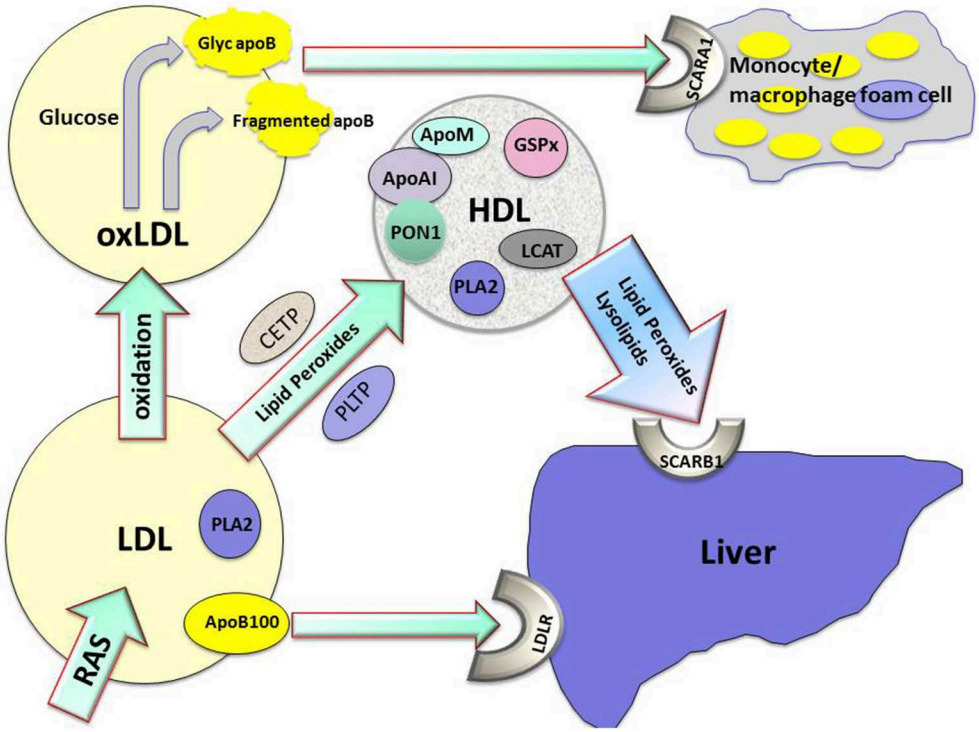

FIGURE 1 | The role played by high density lipoprotein (HDL) in the metabolism of lipid hydroperoxides and lysolipids and protection against atherogenesis. Apo AI, apolipoprotein AI; apoB100, apolipoprotein B100; apoM, apolipoprotein M; CETP, cholesteryl ester transfer protein; glyc apoB, glycated apolipoprotein B; GSPx, glutathione peroxidase; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; oxLDL, oxidized low density lipoprotein; PLA2, phospholipase A2; PLTP, phospholipid transfer protein; PON1, paraoxonase1; RAS, reactive oxygen species; SCARA1, scavenger receptor A1; SCARB1, scavenger receptor B1.

recombinant PON1 for therapeutic use (Bajaj et al., 2014). Interestingly, HDL from avian species, which lacks paraoxonase activity, does not protect human LDL against lipid peroxidation (Mackness et al., 1998). Similarly, *PON1* knockout mice are more susceptible to atherosclerosis and their HDL is less able to prevent the accumulation of lipid peroxides on human LDL (Shih et al., 1998), whereas transgenic rodent models expressing human *PON1* are protected against atherosclerosis (Tward et al., 2002; Zhang et al., 2010).

Epidemiological studies have consistently shown that PON1 activity is independently inversely associated with coronary events (Mackness et al., 2003; Wang et al., 2012). A recent meta-analysis, which considered 47 such studies, reported that PON1 activity was 19% lower in patients suffering from coronary heart disease than in unaffected controls (Wang et al., 2012). Other prospective studies expanded on the negative correlation between PON1 activity and coronary heart disease by also reporting circulating levels of lipid peroxidation products, linking these to PON1 anti-oxidative activity (Bhattacharyya et al., 2008; Karlsson et al., 2015).

A number of medical conditions including diabetes mellitus, chronic kidney disease, familial hypercholesterolaemia and inflammatory arthritides are associated with both decreased serum PON1 activity and increased CVD risk (Soran et al., 2009). PON1 activity is decreased in both type 1 and 2 diabetes (Mackness et al., 2000, 2002) and lower levels are associated with microvascular complications (Mackness et al., 2000, 2002; Hofer et al., 2006).

PON1 has several genetic polymorphisms, the most extensively researched of which is the R192Q variant. This polymorphism has a substantial effect on PON1's capacity to hydrolyze paraoxon and homozygotes and heterozygotes for the R allele are more resistant to parathion (paraoxon is formed from this widely used organophosphate pesticide once it enters the body) than QQ homozygotes (Mackness et al., 2001; Cherry et al., 2002; Wheeler et al., 2004). Other activities of PON1, such as phenyl acetate hydrolysis, which proceed at faster rates, are, however, unaffected by the R192Q polymorphism. In the case of the protective effect of HDL against LDL oxidation, HDL from 192QQ homozygotes is most effective in preventing the accumulation of lipid peroxides on LDL (Mackness et al., 1997; Durrington et al., 2001), but this effect is small in comparison to the huge variation in serum PON1 activity. Nonetheless 192QQ homozygotes have been reported to have reduced CVD risk (Mackness et al., 2001; Wheeler et al., 2004). This inverse association is, however, within the range which could be explained by publication bias, but it does not deny that the wider range of PON1 activities encountered in populations resulting from other genetic and acquired influences are relevant to the development of atherosclerosis. It certainly indicates that the

substrate specificity involved in the antiatherogenic effect of PON1 is not greatly influenced by the R192Q polymorphism.

PON1 and *PON2* genotype have been linked with susceptibility to develop diabetes (Rozenberg et al., 2008), glycaemic control (Hegele et al., 1997), and diabetic microvascular complications (Mackness et al., 2000; Hofer et al., 2006; Wang et al., 2013). It has been suggested that this association reflects a role for oxidation in pancreatic β cell dysfunction or microvascular disease. Alternatively, it might reflect an ability of HDL / PON1 to prevent post-translational protein glycation.

Parenteral administration of partially purified PON1 can ameliorate experimental atherosclerosis. Recombinant PON1 might also be used for this purpose, if its properties can be retained during isolation (Draganov et al., 2005; Bajaj et al., 2014). Intraperitoneal injection of recombinant PON1 in mice increased cholesterol efflux capacity and HDL aryl esterase and lactonase activities, and decreased macrophage mediated LDL oxidation (Rosenblat et al., 2011).

Paraoxonase 2 and Paraoxonase 3

PON2 is almost exclusively found intracellularly, whereas PON3 is also associated with HDL, albeit in lesser quantities than PON1. The primary hydrolytic activity of PON3 is also as a lactonase (Draganov et al., 2005). *PON3* knockout mice are also more susceptible to atherosclerosis (Zhang et al., 2010), but the reason for its evolutionary conservation is currently unclear.

Apolipoprotein AI

ApoAI is essential both for the structure of HDL and the maintenance of the lipid environment in which enzymes such as PON1 and lecithin: cholesterol acyl transferase (LCAT) can operate (Rye and Barter, 2014). It is therefore likely to have a major role in the antioxidant effects of HDL. ApoAI plays a central role in the redox inactivation of lipid hydroperoxides which follows their transfer to HDL. ApoAI also creates a safe environment for the release of lysophospholipids and their subsequent transfer to the liver. Despite experimental evidence that lipid-protein complexes containing only apoAI can protect LDL against oxidation (Karlsson et al., 2015), neither animal models nor human genetic disorders have provided convincing evidence that apoAI's anti-atherogenic effects are independent of changes in HDL levels (Duverger et al., 1996; Plump et al., 1997). Interestingly, apoAI-mimetic peptides create circulating lipid complexes, which associate with other components of HDL such as PON1 (Mishra et al., 2013). This may provide a means of enhancing circulating PON1 activity.

Apolipoprotein AII (apoAII)

Apolipoprotein AII (apoAII)-containing HDL particles tend to be larger and possess less antioxidant activity than those with higher apoAI content (Karlsson et al., 2015). There is evidence from both animal models and human studies to suggest that apoAII might actually suppress PON1 binding to HDL (Litvinov et al., 2012). Mice expressing human apoAII and apoAI are more susceptible to atherosclerosis than those expressing apoAI alone.

Other Apolipoproteins

Other apolipoproteins associated with HDL may act alongside apoAI to inhibit lipid hydroperoxide accumulation. Apolipoprotein E (apoE) appears to display this anti-oxidative activity (Miyata and Smith, 1996), while apolipoprotein M (apoM) has recently been reported to display anti-oxidative functionality in transgenic mice in addition to facilitating PON1 activity (Elsøe et al., 2012; Borup et al., 2015). There is currently little evidence that apolipoprotein J (apoJ) contributes to the anti-oxidative activities of HDL, but it does appear to possess a variety of functions, including endothelial protection.

Myeloperoxidase (MPO)

Cellular systems contributing to oxidative stress *in vivo* include MPO, NADPH oxidase, nitric oxide synthase, and lipoxygenase (Karlsson et al., 2015). It has recently been proposed that MPO and PON1 form a ternary complex with HDL, where the opposing activities of MPO and PON1 determine its oxidation state and whether HDL is pro- or anti-inflammatory/atherogenic (Huang et al., 2013). The ratio between these enzyme activities has also been proposed as a marker of HDL functionality and to predict coronary risk (Haraguchi et al., 2014).

Glutathione Peroxidase (GSPx)

Although, its levels do not appear to affect coronary heart disease risk, GSPx is found associated with HDL, where it has the ability to reduce lipid hydroperoxides (Karlsson et al., 2015). Similarly, trypanosome lytic factor present in higher density HDL also exhibits peroxidase activity and may contribute to the anti-oxidative properties of HDL (Karlsson et al., 2015).

Phospholipase A2

Most PLA2 is associated with LDL, where its activity is an independent risk factor for coronary heart disease (Rosenson and Hurt-Camejo, 2012). There is however, no evidence that the minor fraction of PLA2 activity on HDL is pro-atherogenic. Furthermore, PLA2 has overlapping activity with PON1 and it remains unclear just how much of the hydrolysis of platelet activating factor by HDL is due to PLA2 and how much to PON1 (Rodrigo et al., 2001). PLA2 on HDL is likely to contribute anti-oxidative activity by the same mechanism as PON1, by hydrolyzing lipid hydroperoxides. This activity, which would be pro-atherogenic on LDL in the presence of apoB, may be antiatherogenic in the environment provided by HDL.

Lecithin: Cholesterol Acyl Transferase

Similarly, there is currently limited evidence to support a role for LCAT in the antioxidative activity of HDL (Holleboom et al., 2012), but its association with HDL and, like PON1 and PLA2,

its role in generating lysophospholipids, does contribute to the hypothesis that HDL provides a safe place to release lysolipids otherwise potentially damaging to cell membranes and other lipoproteins.

Cholesteryl Ester Transfer Protein (CETP) and Phospholipid Transfer Protein (PLTP)

The antioxidant activity of HDL occurs following the transfer of lipid hydroperoxides to HDL. Experimentally no additional facilitator of transfer to HDL is required, but some CETP and PLTP are likely to remain in physical association with HDL after its isolation. CETP can accelerate the transfer of both cholesteryl ester and phospholipid hydroperoxides (Christison et al., 1995). CETP and/or PLTP may thus be important for the anti-oxidative effect of HDL *in vivo*. Like PON1, PLTP is found predominantly in small, dense HDL, where it is able to interact with apolipoproteins implicated in anti-oxidative function, including apoAI, apoAII, and apoJ (Karlsson et al., 2015).

Conclusion

The capacity of HDL to protect LDL against oxidative modification is considerable, but its potential therapeutic use to prevent atherosclerosis is as yet unfulfilled. The interaction of lipids with apoAI in HDL provides a lipoprotein particle capable of acquiring potentially toxic lipids and holding them in an environment where they may be safely hydrolyzed and from which they may be released to the liver for elimination. PON1, PLA2, and LCAT are present at higher concentrations in small, dense, protein-rich HDL (Karlsson et al., 2015), and HDL particles are therefore heterogeneous in their anti-oxidative capacity. PON1 is likely to be critical to the antioxidative capacity of HDL, but is likely to require a lipid environment to support its activity. Separation of HDL from PON1 disrupts this and the necessary environment is only imperfectly present with the currently available water-soluble recombinant forms of PON1. Acting together on HDL, PON1, apoAI, apoM, and PLA2 in conjunction with CETP and other lipid transfer proteins probably create a system with both antioxidative and antiglycative properties (see **Figure 1**).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. High-Density Lipoprotein Functionality in Type 1 Diabetes Mellitus

Abstract

Introduction

Type 1 diabetes mellitus (T1DM) is associated with increased cardiovascular risk, and high-density lipoprotein (HDL) functionality may be an important determinant of this. HDL cholesterol levels are normal or even high in T1DM prior to the development of nephropathy, but do not seem to protect against atherosclerosis as might be expected, suggesting HDL dysfunction.

Method

In this cross-sectional study we explore assays of HDL functionality in patients with T1DM (n = 91) and healthy volunteers (n = 104), and their relationships with biomarkers of cardiovascular risk and surrogate outcomes, including coronary artery calcification scoring and carotid doppler studies, and quantitive assessments of neuropathy.

Results

Although HDL cholesterol was significantly higher in T1DM than in healthy controls, no difference was observed in apolipoprotein (apo) AI levels or cholesteryl ester transfer protein (CETP) activity. Cholesterol efflux capacity and paraoxonase-1 (PON1) activity were significantly lower in T1DM compared to healthy controls. Cholesterol efflux capacity was weakly correlated with apo AI in healthy controls, but not in T1DM. Apo E levels were similar in healthy controls and T1DM, but the concentration of apo E associated with HDL was significantly lower in T1DM. Glycated apo B and oxidized low-density lipoprotein were significantly higher in T1DM, and the negative correlation between apo AI and these markers seen in healthy controls was lost in T1DM. Apo M, myeloperoxidase activity and mass were significantly lower in T1DM than healthy controls. HDL-associated apo CIII and vascular cell adhesion molecule 1 were significantly higher in T1DM and the negative correlation between apo AI and high-sensitivity C-reactive protein seen in healthy controls was also lost. The assessed measures of HDL functionality did not demonstrate any significant correlation with measures of subclinical atherosclerosis (coronary artery calcification score and carotid-intima media thickness) in patients with T1DM, supporting HDL dysfunction. There was a weak but statistically significant negative correlation between corneal confocal microscopy parameters and HDL cholesterol levels but no correlation with markers of HDL functionality.

Discussion

This study offers new insight into HDL dysfunction and its relationship with macrovascular and microvascular complications in T1DM.

Introduction

Diabetes mellitus is associated with a considerably increased risk of premature atherosclerosis, particularly coronary heart disease (CHD) and peripheral arterial disease [14, 15]. Early studies of cardiovascular mortality in type 1 diabetes (T1DM) suggested that risk only significantly increases after the development of nephropathy, which coincides with a marked deterioration of the lipid profile and blood pressure [165], but T1DM is associated with a relative risk of 3.0 for women and 2.3 for men even in the absence of microalbuminuria [167].

Hypertriglyceridaemia may occur in T1DM, but high-density lipoprotein (HDL) cholesterol levels are often normal or even high unless glycaemic control is poor or nephropathy has developed [16]. The apparently normal serum cholesterol concentrations observed in T1DM have led to the widespread erroneous belief that glycaemia alone might be responsible for the observed high CHD rates. However, the relatively normal cholesterol levels of T1DM hide an atherogenic lipid profile, with dysfunctional HDL and qualitative and kinetic lipoprotein abnormalities [207-209].

Although HDL levels are a strong biomarker for assessing cardiovascular risk, emerging evidence suggests that HDL functionality is more important than cholesterol cargo [12]. Assays of HDL functionality offer an alternative to plasma HDL cholesterol concentrations, but their reliable assessment remains challenging [435]. Promising biomarkers of HDL functionality and thus cardiovascular risk include plasma myeloperoxidase (MPO), paraoxonase-1 (PON1) [63, 64], apolipoprotein AI (apo AI), lecithin-cholesterol acyltransferase (LCAT), lipoprotein phospholipase A2 (lp-PLA2) and serum amyloid A (SAA) [12, 65].

The increased cardiovascular risk associated with T1DM remains poorly understood, and is paradoxical to the anticipated effects of the higher HDL cholesterol levels observed in patients with T1DM [436]. HDL dysfunction is suggested by the observation that high HDL cholesterol levels do not always protect against CHD [11, 130]. Exogenous insulin upregulates lipoprotein lipase in T1DM, increasing the production of small HDL particles [210, 211], frequently to higher than normal HDL cholesterol levels [212]. HDL cholesterol in T1DM has thus not arisen physiologically.

Cholesteryl ester is transferred from other lipoproteins into the enlarged circulating pool of triglyceride-rich lipoproteins by cholesteryl ester transfer protein (CETP), and the rate of transfer is reported to be increased in T1DM [202]. CETP can accelerate the transfer of both cholesteryl ester and phospholipid hydroperoxides [433]. It may therefore be important for the anti-oxidative effect of HDL *in vivo*. With the development of nephropathy, increased

catabolism of smaller HDL particles likely combines with increased cholesteryl ester activity to lower HDL cholesterol levels [165, 216]. Glycation of HDL further enhances its catabolism [217]. T1DM thus eventually leads to both HDL dysfunction and reduced concentrations [194].

Dysfunctional HDL is pro-inflammatory and contains oxidized phospholipids and lysophospholipids, as well as pro-inflammatory proteins, such as SAA. HDL may be dysfunctional in its capacity to protect low-density lipoprotein (LDL) against atherogenic modification. Glycated LDL may be more susceptible to oxidation or itself represent an atherogenic modification [207, 208].

HDL-associated PON1 is important in impeding atherogenic modifications of LDL [368, 369, 379]. Epidemiological studies have consistently shown that PON1 activity is independently inversely associated with coronary events [390, 391]. The ratio between MPO and PON1 enzyme activities has also been proposed as a marker of HDL functionality and to predict coronary risk [427].

Apo AI is essential both for the structure of HDL and the maintenance of the lipid environment in which enzymes such as PON1 and LCAT can operate [421]. It is therefore likely to have a major role in the antioxidant effects of HDL. Apo AI plays a central role in the redox inactivation of lipid hydroperoxides which follows their transfer to HDL, and there is experimental evidence that only lipid-protein complexes containing apo AI can protect LDL against oxidation [394]. Apolipoprotein M (apo M) has also recently been reported to display anti-oxidative functionality in transgenic mice in addition to facilitating PON1 activity [424, 425].

In this cross-sectional study we explore recognized biomarkers of HDL functionality in patients with T1DM, and their relationships with biomarkers of cardiovascular risk including coronary artery calcification scoring and carotid doppler studies, and quantitative assessments of neuropathy.

Method

Patients with T1DM (n = 91) were recruited through Central Manchester University Hospitals NHS Foundation Trust, University Hospital of South Manchester, Lancashire Teaching Hospitals NHS Foundation Trust, Bradford Institute for Health Research, and through the Diabetes Research Network 'Consent for Approach' Database (Help Diabeates®). The study was performed according to the Declaration of Helsinki and was approved by the National Research Ethics Service.

All participants were reviewed to assess basic demographic details, type and duration of diabetes, co-morbid conditions, known complications, insulin and lipid-lowering medication use, and the use of tobacco and alcohol. Patients with a history of coronary heart disease were excluded. Measurements were taken for height, weight and blood pressure, a fasting blood sample taken and urinalysis performed. Patients with T1DM were divided into 2 cohorts according to recruitment site: Cohort 1 (n = 51) also had assessment of carotid intima media thickness (CIMT) and atheroma burden by carotid doppler studies and assessment of nerve function and structure by corneal confocal microscopy (CCM); Cohort 2 (n = 40) also had assessment of Coronary Artery Calcification (CAC) by cardiac computed tomography (CT).

Healthy controls (n = 104) were recruited through the University of Manchester, Central Manchester University Hospitals NHS Foundation Trust and Barlow Medical Centre. Volunteers with any history suggestive of coronary heart disease, vascular insufficiency or diabetes, using any lipid lowering drugs (including omega fatty acid supplements) were excluded.

Demographic and clinical characteristics of the study participants are shown in Table 6.1. 55 (60.4%) of the patients with T1DM were on multiple dose insulin injection therapy and 36 (39.6%) received their insulin by continuous subcutaneous insulin infusion. 12 (13.2%) were receiving statin therapy and 7 (7.7%) were receiving angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers.

Venous blood samples were collected after overnight fasting and serum and EDTA-plasma isolated by centrifugation at 2000 x G for 15 minutes at 4^{o}C within 2 hours of collection. Samples were aliquoted, anonymized and stored at 4^{o}C until all clinical laboratory testing was complete. Remaining samples were frozen to -80^{o}C.

Glycated haemoglobin (HbA1c) was analyzed by high-performance liquid chromatography on a VARIANT II TURBO Hemoglobin Testing System (Bio-Rad, Hercules, CA, USA). Glucose, cholesterol and triglycerides were determined by enzymatic hydrolysis and precipitation using glucose oxidase phenol 4 aminoantipyrine (GOD-PAP), cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) and glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) methods respectively (ABX Horiba-UK). HDL cholesterol was measured by a direct second-generation homogeneous method (Roche Diagnostics) and LDL cholesterol was estimated using the Friedewald Formula. Apo AI and apolipoprotein B (apo B) were measured immunoturbidimetrically. A Cobas Mira autoanalyzer (ABX Horiba-UK, Northampton, UK) was used for all of these assays.

Apolipoprotein-CIII (apo CIII) (Sigma-Aldrich, St. Louis, MO, USA), apo M (Holzel Diagnostika Handels GmbH, Cologne, Germany), MPO Mass (R&D Systems Europe, Abingdon, UK), Oxidized LDL (Mercodia AB, Uppsala, Sweden), SAA (ThermoFisher Scientific, Loughborough, UK) and Vascular Cell Adhesion Molecule 1 (VCAM-1) (R&D Systems Europe, Abingdon, UK) were measured by quantitative sandwich immunoassay. Glycated apo B was measured by indirect competitive ELISA (Exocell, Philadelphia, PA, USA). Apolipoprotein E (apo E) was measured by in-house high-sensitivity sandwich ELISA. Cholesterol efflux capacity was assessed in a method based upon that of Khera *et al* [76]. All assays were run in duplicate. CETP Activity was assessed using ^{3}H-labelled cholesterol as a substrate. MPO activity was assessed using a colourimetric activity assay kit (Sigma-Aldrich, St. Louis, MO, USA). PON1 activity was determined by a semi-automated micro-titre plate method using paraoxon (*O,O*-Diethyl *O*-(4-nitrophenyl)phosphate) as a substrate.

The presence of microalbuminuria was assessed by measurement of albumin-to-creatinine ratio in a random spot urine collection [437].

Patients with T1DM were evaluated for the presence of diabetic sensory neuropathy (DSN) using a modified neuropathy disability score (NDS) with assessment of vibration perception using a 128-Hz tuning fork, ankle reflex testing, temperature differentiation and pinprick testing. A score of 0 was given for a normal response and 1 for an abnormal response for each individual test component (except ankle reflex testing, where a score of 1 indicates presence with reinforcement, and 2 complete absence). Thus the maximum score is 10, with an NDS of ≥ 3 indicative of peripheral neuropathy [30, 31]. The presence of large fibre neuropathy was evaluated by vibration perception threshold testing (VPT) using a Neurothesiometer (Horwell; Scientific Laboratory Supplies, Nottingham, UK) and nerve conduction studies for selected patients (Dantec Dynamics, Bristol, UK). The presence of small fibre neuropathy was evaluated by quantitative sensory testing using a TSA-II NeuroSensory Analyzer (Medoc, Ramat-Yishai, Israel) and corneal confocal microscopy (CCM) using a Heidelberg Retinal Tomograph III Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany). Neuropathy was diagnosed through CCM according to known age-adjusted normative values for corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD) and corneal nerve fibre length (CNFL) [32].

40 patients with T1DM underwent CAC Score determination by electron-beam CT using an automated program based on the Agatson method as a screening tool to identify subclinical atherosclerosis [27]. A CAC Score >10 was considered significant [28]. The carotid arteries were imaged in the remaining patients with T1DM with a Siemens Sequoia ultrasonography system (Siemens Medical Solutions, Mountain View, CA) with an 8- to 15-MHz linear array transducer. Examination included measurement of common and internal carotid artery flow velocities and Carotid Intima-Media thickness (CIMT) at each of 3 scan planes. Here a mean

CIMT of 0.06 cm was considered significant [29].

The clinical and lipid data were merged and clinical baseline characteristics and HDL-related parameters compared between groups by univariate and multivariate linear regression models to control for baseline differences between groups. Normal distribution was tested for all data with the Kolmogorov-Smirnov, D'Agostino and Pearson omnibus and Shapiro-Wilk normality tests. A Bonferroni correction to account for multiple testing in multivariate model building was applied, with a significance level of 0.05 (Appendix Table 6.4). All analyses were performed with SPSS statistical software, version 22.0 (IBM, Zurich, Switzerland). Patients with T1DM were subdivided according to the presence of microalbuminuria. Significance was assessed with Student paired and unpaired, two-tailed t tests. Simple linear correlations were calculated by determining the Pearson correlation coefficient *R*.

Results

Lipid Profile

As expected, patients with T1DM had a higher fasting glucose (9.19 ± 4.41 *vs.* 4.50 ± 0.66 mmol/l, $P < 0.0001$). The routine lipid profile appeared to be less adverse in patients with T1DM (Figure 6.1), with total and LDL cholesterol significantly higher in healthy volunteers (5.28 ± 1.05 *vs.* 4.78 ± 0.80 mmol/l, $P = 0.0008$ and 3.24 ± 0.82 *vs.* 2.62 ± 0.66 mmol/l, $P < 0.0001$ respectively), and HDL cholesterol significantly higher in T1DM (1.70 ± 0.45 *vs.* 1.42 ± 0.35 mmol/l, $P < 0.0001$). Triglycerides were also significantly lower in patients with T1DM (1.04 ± 0.51 *vs.* 1.36 ± 0.84 mmol/l, $P = 0.003$).

These apparently positive effects on the lipid profile were less clear following the development of microalbuminuria (≥ 3.5 mg/mmol in females or ≥ 2.5 mg/mmol in males), with total and LDL cholesterol still lower in patients with T1DM and microalbuminuria (4.31 ± 0.90 mmol/l, $P = 0.03$ and 2.30 ± 0.81 mmol/l, $P = 0.009$ respectively) compared to healthy controls, but HDL cholesterol non-significantly lower (1.37 ± 0.29 mmol/l) and mean triglyceride levels increased to 1.39 ± 0.62 mmol/l. 3 of 6 (50%) patients with T1DM and microalbuminuria were treated with statin therapy compared with 13 of 85 (15%) of patients with T1DM but no microalbuminuria.

There was a strong correlation between HDL and apo AI both for patients with T1DM and healthy controls ($R = 0.86$, $P < 0.0001$ and $R = 0.81$, $P < 0.0001$ respectively – Figure 6.2). Although HDL cholesterol was significantly higher in patients with T1DM, no difference was observed in apo AI (1.57 ± 0.30 *vs.* 1.55 ± 0.28 g/L) between the T1DM and healthy control cohorts. When healthy volunteers were matched according to HDL cholesterol, apo AI levels

were significantly lower in patients with T1DM compared to healthy controls (1.57 ± 0.30 *vs.* 1.73 ± 0.25 g/L, *P* = 0.01).

Reverse Cholesterol Transport and Lipid Transfer

We did not find any difference in CETP activity between patients with T1DM and healthy controls. HDL cholesterol levels did not predict CETP activity, cholesterol efflux capacity or PON1 activity in healthy controls or patients with T1DM. Initial analysis also did not demonstrate any effect of T1DM on cholesterol efflux capacity, but when patients with T1DM on statin therapy were excluded from the analysis and values adjusted for HDL, cholesterol efflux capacity was significantly reduced in patients with T1DM compared to healthy controls (10.31 ± 3.00 *vs.* 12.54 ± 5.35%, *P* = 0.02). Cholesterol efflux capacity was weakly correlated with apo AI in healthy controls (*R* = 0.27, *P* = 0.013), but not in patients with T1DM. In contrast to reports from healthy volunteers there was only a weak negative correlation between cholesterol efflux capacity and CIMT (*R* = -0.13, *P* = NS) in patients with T1DM.

Apo E levels were similar in healthy controls and patients with T1DM, but the concentration of apo E associated with HDL was significantly lower in patients with T1DM (24.74 ± 12.36 *vs.* 35.80 ± 14.96 mg/l, *P* < 0.0001) (Figure 6.3).

Protection against Oxidation / Glycation

The proportion of LDL having undergone atherogenic modification was significantly higher in patients with T1DM compared to healthy controls, with glycated apo B accounting for 3.84 ± 0.77 of the total apo B in T1DM compared with 2.30 ± 1.57% in healthy controls, *P* < 0.0001 and the proportion of oxidized LDL also significantly higher in T1DM at 15.66 ± 3.22 compared to 11.71 ± 5.64 μg/mmol in healthy controls, *P* < 0.0001. There was a negative correlation between apo AI and oxidized LDL levels both in healthy controls and in T1DM (*R* = -0.60, *P* < 0.0001, and *R* = -0.39, *P* = 0.003 respectively), but the weak negative correlation between apo AI and glycated apo B observed in healthy controls (*R* = -0.26, *P* = 0.017) was lost in patients with T1DM.

PON1 activity was significantly lower in patients with T1DM compared to healthy controls (118.67 ± 73.79 *vs.* 164.30 ± 97.65 nmol/ml/min, *P* = 0.0003) (Figure 6.4). This difference persisted through all adjustments. There was a weak negative correlation between PON1 activity and CAC score (*R* = -0.26, *P* = 0.05) but both CAC score and CIMT were predicted more by age (*R* = 0.37, *P* = 0.009 and *R* = 0.62, *P* < 0.0001 respectively) and duration of diabetes (*R* = 0.38, *P* = 0.007 and *R* = 0.48, *P* < 0.001 respectively).

When patients with T1DM on statin therapy were excluded from the analysis apo M was also significantly lower in patients with T1DM compared to healthy controls (427.8 ± 185.4 *vs.* 535.1 ± 150.1 μmol/L, *P* = 0.002).

MPO activity and mass were significantly lower in patients with T1DM compared to healthy controls (4.63 ± 2.14 *vs.* 7.50 ± 5.77 mU/ml, *P* = 0.001, and 449.63 ± 197.89 *vs.* 755.78 ± 506.808 pg/ml, *P* < 0.0001). These differences persisted through all adjustments. The association between apo AI and MPO activity appeared to be affected by the presence of diabetes, with healthy controls demonstrating a weak negative correlation (*R* = -0.22, *P* = 0.039) but patients with T1DM demonstrating a weak positive correlation (*R* = 0.27, *P* = 0.027). Similarly, there was little relation between MPO activity and cholesterol efflux capacity in patients with T1DM but a moderate negative correlation in healthy controls (*R* = -0.45, *P* < 0.0001).

Protection against Inflammation

Further to the relationships described above, apo AI concentrations showed (weak) negative correlation with markers of inflammation including hsCRP (*R* = -0.29, *P* = 0.009) in healthy controls, but there did not appear to be any relationship in patients with T1DM. Plasma VCAM-1 concentrations were significantly higher in patients with T1DM compared to healthy volunteers (440.61 ± 115.75 *vs.* 325.98 ± 42.59 ng/ml). No difference in SAA concentrations was observed between patients with T1DM and healthy controls although there was a moderate correlation between SAA and the microalbumin creatinine ratio (*R* = 0.32, *P* = 0.001), and SAA was significantly higher in patients with T1DM and microalbuminuria than patients with T1DM without microalbuminuria (*P* = 0.02). The proportion of apo CIII associated with HDL was higher in patients with T1DM and microalbuminuria.

Post hoc analyses considering the effect of T1DM according to gender demonstrated a greater reduction in the proportion of apo E associated with HDL (*P* = 0.0004 *vs.* 0.016), and less resistance to apo B glycation (*P* < 0.0001 *vs.* 0.004) or oxidation (*P* = 0.0003 *vs.* 0.012) in women compared to men.

The presence of microalbuminuria in patients with T1DM was associated with suboptimal glycaemic control, hypertension, neuropathy (Table 6.2), lower HDL cholesterol and higher triglyceride levels, but no significant effect was detected on markers of HDL functionality or macrovascular complications as assessed by CAC scoring or carotid doppler measurements (Table 6.3).

There was a strong correlation between parameters assessed by CCM (*R* = 0.77, *P* < 0.0001 for CNFD *vs.* CNBD, *R* = 0.88, *P* < 0.0001 for CNFD *vs.* CNFL and *R* = 0.86, *P* < 0.0001 for CNBD *vs.* CNFL) and all values were significantly lower in patients with T1DM compared to

known normative measurements. Despite the cross-sectional design of this study, there was a weak negative correlation between HbA1c and CCM parameters, with no significant correlation between either glycated apo B or glucose and CNFD, CNBD or CNFL. There was a weak negative correlation between HDL cholesterol levels and all CCM parameters (R = -0.18 for CNFD, R = -0.35 for CNBD (P = 0.007) and R = -0.19 for CNFL) but no correlation between CCM parameters and cholesterol efflux capacity, CETP or PON1 activity. Factors predicting neuropathy in this cohort of patients with T1DM are shown in Appendix Table 6.6.

Interestingly, alcohol consumption within the T1DM cohorts was associated with increased HDL cholesterol (1.79 ± 0.46 *vs.* 1.42 ± 0.35 mmol/l, P = 0.0009 and apo AI (1.66 ± 0.31 *vs.* 1.39 ± 0.25 g/L, P = 0.005), but no difference in markers of HDL functionality including cholesterol efflux capacity, PON1, CETP and MPO.

Discussion

Risk

Cardiovascular risk in T1DM is strongly related to glycaemic control, nephropathy, and hypertension, and is significantly increased compared with normoglycaemic subjects [257]. While features such as the presence of nephropathy or retinopathy identify higher risk groups, the use of other biomarkers of risk and likely need for enhanced treatment are often not appreciated.

The risk of atherosclerotic cardiovascular disease in patients with T1DM is greater at any given level of serum cholesterol and its association with hypertriglyceridaemia is stronger than in the general population [181]. Importantly, there is strong and convincing evidence that cholesterol-lowering therapy significantly reduces CHD in patients both with and without diabetes [182-184].

Lipid Profile

This study confirms the lipoprotein changes with diabetes demonstrated in the Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications cohort [19]. With the development of nephropathy total and LDL cholesterol, intermediate density lipoproteins, triglycerides, and apo B levels rise, and HDL cholesterol and Apo AI levels fall [438].

Glycaemic control may not determine HDL function, but it is well recognized that HbA1c does not fully reflect glucose control in T1DM [439]. Oscillating glucose levels in T1DM are associated with increased oxidative stress in patients with both good and suboptimal glycaemic control [322]. The functional capacity of HDL is likely impaired by irreversible

post-translational modifications caused by hyperglycaemia and oxidative stress [440]. The current study demonstrated significant reductions in cholesterol efflux capacity and HDL anti-oxidative function in T1DM compared to healthy controls.

Differences in HDL-associated enzyme or lipoprotein distribution could explain residual CHD risk in an otherwise favourable lipid profile. HDL-mediated atheroprotection appears pleiotropic in nature. HDL particles contain antioxidants and enzymes involved in plasma lipid metabolism, including PON1, CETP, LCAT and phospholipid transfer protein, each of which contributes to HDL composition and function [441]. HDL concentration, composition and metabolism differ considerably in patients with T1DM [65]. Determining HDL function may identify patients with normal or even high HDL cholesterol at high risk of CVD.

The HDL particle distribution is abnormal in T1DM, with a relative reduction in the fraction of large HDL particles, which are believed to be cardioprotective [442]. Apo AI is present in almost all HDL particles [14]. It is the major structural protein in HDL, and thus has a significant role in determining the structure and composition of HDL [443]. A *post-hoc* analysis of two prospective studies demonstrated that high levels of HDL cholesterol are associated with increased cardiovascular risk unless accompanied by an elevated apo AI [444]. The observation here that increased levels of HDL cholesterol are not accompanied by increased apo AI in T1DM may therefore represent increased cardiovascular risk.

Reverse Cholesterol Transport

The capacity of HDL cholesterol to promote cholesterol efflux from macrophages is a recognized metric of HDL function and has recently been shown to have a strong inverse relationship with both CIMT and the presence of angiographically confirmed coronary artery disease, independent of HDL concentration [76, 77]. Cholesterol efflux from peripheral tissues to HDL particles is a complex process that depends on cellular cholesterol status, apo AI and apo E concentration and distribution, HDL composition, and the expression of membrane cholesterol transporters [445]. Glycation of HDL takes place mainly on Apo AI, diminishing HDL receptor binding and its ability to stabilize ATP-binding cassette transporter (ABCA1) [372]. This would be expected to impair reverse cholesterol transport. Although initial analysis here did not demonstrate any effect of T1DM, cholesterol efflux capacity was significantly reduced in patients with T1DM compared to healthy controls when patients with T1DM on statin therapy were excluded from the analysis and values adjusted for HDL cholesterol. The observed decreased strength of correlation between cholesterol efflux and apo AI in patients with T1DM, similar to that previously reported in association with cardiovascular disease [446], is suggestive of HDL dysfunction.

Oxidative modification of HDL by MPO leads to loss of ABCA1-mediated cholesterol efflux activity [447]. The major initial acceptor for cholesterol excreted by cells is pre-β HDL;

other lipid-free apolipoproteins and small HDL particles can also promote cholesterol efflux by ABCA1. The major acceptor for free cholesterol export by ABCG1 is spherical HDL [448].

CIMT is a good surrogate marker of atherosclerosis and an independent predictor of cardiovascular events [449]. The capacity of HDL to promote cellular cholesterol efflux in an *ex vivo* model has been reported to correlate more closely with CIMT than HDL cholesterol concentration [76]. This remains true even for the patients with T1DM and reduced cholesterol efflux capacity reported here.

Apo E is a structural component of several lipoproteins including HDL [450]. Apo E-rich HDL is a particularly good acceptor of excess cellular cholesterol and prevents LDL binding to proteoglycans in the vessel wall [451]. The observation here that the concentration of apo E associated with HDL was significantly lower in patients with T1DM than in healthy volunteers is likely to be relevant to both cholesterol efflux and other routes of lipid transfer. We also observed higher levels of glycated apo E in patients with T1DM, which could also contribute to impaired cholesterol efflux capacity and HDL anti-oxidative functions [423].

Lipid Transfer

CETP mediates neutral lipid transfer between HDL and apo B-containing lipoproteins, favouring the formation of smaller HDL particles [452]. Increased CETP activity has previously been reported in both T1DM and T2DM [453]. We did not observe any difference in CETP activity between patients with T1DM and healthy controls. It should however be noted that CETP activity primarily depends on the availability of both HDL cholesterol and triglycerides [454]. Over-expression of lecithin-cholesterol acyltransferase increases the risk of atherosclerosis despite elevated levels of HDL cholesterol and apo AI [455].

Protection against Oxidation / Glycation

There is evidence for multiple antioxidant mechanisms of HDL [456]. Reductions in these anti-oxidative effects have been reported in patients with diabetes [192, 213]. We previously reported that HDL can protect LDL against glycation as well as oxidation [71]. Glycated LDL is present in the circulation under physiological conditions and at higher concentrations in diabetes, suggesting that HDL's anti-glycative effects are also reduced [205]. The proportion of glycated apo B is doubled even in reasonably well-controlled diabetes [187]. Here we have demonstrated significant increases in the proportion of both glycated and oxidized LDL in patients with T1DM, consistent with impaired anti-oxidative and anti-glycative activities.

PON1 activity in serum is inversely related to the risk of cardiovascular disease [390]. Apo AI stabilizes PON1, stimulates its hydrolytic activities, and facilitates the interaction of

PON1 with its natural substrates [457]. Under pathological conditions such as T1DM, PON1 dissociates from HDL to the lipoprotein-free serum fraction, where it is less biologically active [458]. The observation here of both reduced apo AI and PON1 activity is therefore highly significant. Low serum PON1 activity, independent of genotype, has also been reported to be associated with microvascular complications [396]. PON1 activity is further reduced in patients with microalbuminuria [459]. Low serum PON1 activity in T1DM has been implicated in both reduced HDL anti-oxidative and anti-glycative functionality [71].

In previous studies investigating the relationship between PON1 activity and CAC score, there was no association in the Coronary Artery Risk Development in Young Adults Study [460]. The anti-oxidant activity of PON1 may be of particular relevance in T1DM, where increased oxidative stress associated with glucose fluctuations has been implicated in the associated increased risk of CHD [341]. Low PON1 activity in T1DM may contribute to this increased susceptibility by reducing the ability of HDL to impede lipoprotein oxidation. PON1 activity and concentration were previously reported to be lower in patients with T1DM independent of known polymorphisms for low PON1 activity [396]. The low activity of PON1 has been postulated to be due to glycosylation but the absence of any correlation between PON1 activity and HbA1c level is more suggestive of reduced synthesis or increased catabolism. Increased oxidative stress has been shown to reduce PON1 synthesis in *ex vivo* models and T1DM is associated with increased oxidative stress [341]. PON1 is also not associated with all HDL particles. Low PON1 activity in T1DM may therefore be associated with reduced HDL anti-oxidative capacity, which may explain the increased susceptibility of patients with T1DM to develop CHD [461].

Apo M associated with HDL has also been reported to bind oxidized phospholipids and increase the anti-oxidant effect of HDL [424]. The significantly lower levels of Apo M observed in patients with T1DM in this study are therefore consistent with reduced anti-oxidant capacity.

MPO is fundamentally involved in the development of atherosclerotic lesions [462]. Its catalytic activity is governed largely by the availability of its substrate hydrogen peroxide. Although the observation of significantly lower MPO mass and activity in patients with T1DM reported here may seem surprising, higher levels may only be associated with increased risk in patients with low HDL cholesterol levels [463]. Other studies have also reported significantly lower MPO levels in patients with T1DM when compared to healthy volunteers [464]. Anti-MPO antibodies have been detected in patients with T1DM, associated with a state of chronic neutrophil activation [465], which would be expected to increase vascular inflammation.

Protection against Inflammation

The anti-inflammatory effects of HDL may be mediated by PON1 and phospholipase A2 associated with HDL [403]. HDL can also ameliorate endothelial dysfunction by enhancing nitric oxide synthesis, and by decreasing the expression of adhesion molecules on endothelial cells, thereby reducing inflammation [466]. In this study the negative correlation between apo AI concentration and markers of inflammation including hsCRP seen in healthy controls was not found in patients with T1DM. Reductions in the anti-inflammatory effects of HDL have previously been reported in patients with diabetes, alongside an impaired ability of HDL to counteract the inhibition of endothelium-dependent vasorelaxation by oxidized LDL [192, 213]. Dysfunctional HDL associated with lower PON1 activity has previously been linked to increased endothelial VCAM-1 expressions and reduced NO production by an oxidized LDL receptor (LOX-1) dependent pathway in patients with CHD [467]. Here we present evidence for similar dysfunction in T1DM. The absence of PON1 has previously been shown to be associated with over-expression of adhesion molecules, suggesting an anti-inflammatory role for PON1 [468].

Phospholipids and sphingolipids play a major role in HDL functionality, and altered concentrations of some sphingolipids in HDL can predict CHD independently of HDL cholesterol levels [469]. An abnormal phospholipid or sphingolipid profile in HDL may contribute to HDL dysfunction in T1DM [470]. Sphingosine-1-phosphate (S1P) mediates the activation of endothelial nitric oxide synthesis by HDL, in addition to anti-inflammatory effects [471]. Total plasma and HDL-associated S1P concentrations are lower in T1DM. HDL S1P is carried by apo M [470].

Dysfunctional HDL

In T1DM, HDL loses its protective functions and might contribute to inflammatory processes that promote CHD [472]. Statins have been suggested to attenuate the pro-inflammatory effects of HDL and statin therapy is known to lower SAA levels [473]. Pro-inflammatory HDL contains increased levels of caeruloplasmin and SAA, and decreased levels of apo AI, PON1 and lp-PLA2 [474], and has been associated with increased CIMT and an increased risk of adverse clinical outcomes [74]. SAA might influence HDL uptake of excess tissue cholesterol by displacing apo AI from HDL, and by modulating LCAT activity [475]. Studies investigating serum SAA in T1DM have been inconclusive. HDL-associated SAA is increased in T1DM. The apparently lower sensitivity of serum SAA compared to HDL-associated SAA may reflect differences between acute and chronic inflammation, where SAA associated with HDL is predicted to be more stable [476].

Apo CIII is a pro-inflammatory protein that resides on the surface of very low-density lipoprotein, LDL and HDL particles [477]. The presence of apo CIII in HDL has been used to identify people at risk of future CHD [478]. In this study we observed an increased

proportion of apo CIII associated with HDL in patients with T1DM and microalbuminuria, suggesting HDL dysfunction.

In keeping with the theory of HDL dysfunction, the assessed measures of HDL functionality did not demonstrate any significant correlation with measures of subclinical atherosclerosis (CAC score and CIMT) in patients with T1DM. Factors predicting subclinical atherosclerosis in this cohort of patients with T1DM are shown in Appendix Table 6.5.

Future work

This study confirms previous suggestions that HDL functionality is impaired in T1DM even in the presence of good glycaemic control [479]. With respect to the smaller than anticipated effect on cholesterol efflux observed in patients with T1DM, it should be noted that HDL isolated by ultracentrifugation and HDL derived from apo B precipitation are not equivalent; HDL isolated by ultracentrifugation includes HDL-associated apo E but may not represent pre-β HDL. It should also be noted that the J774 cells used in the assessment of cholesterol efflux do not express apo E [480]. Apo E mediated efflux may thus be underestimated in this study.

The findings reported here also raise the possibility that a substantial portion of the protective effect of HDL may be due to functions beyond reverse cholesterol transport [12]. This work might be expanded by consideration of other known HDL functions, such as HDL-mediated phosphorylation of endothelial nitric oxide synthase or HDL-mediated nitric oxide production and their effects on endothelial function. We will also assess the capacity of HDL from patients with T1DM to inhibit LDL oxidation and glycation *in vitro,* and explore HDL particle distribution in these cohorts, as both HDL particle number and size have been linked to cardiovascular risk [481].

A weakness in the present study is its cross-sectional, observational nature, and it is therefore difficult to draw causal relationships. This study can also not address the extent to which qualitative and kinetic lipoprotein abnormalities cause or result from increased albumin excretion. Another limitation is the small sample size, especially in the microalbuminuria group.

	Patients with T1DM (n = 91)	Healthy Controls (n = 104)
Male / Female (%)	42 (46.1) / 49 (53.8)	44 (42.3) / 60 (57.7)
Age (years)	41.2 ± 13.5	36.8 ± 10.6
Duration of Diabetes (years)	20.8 ± 12.5	-
BMI (kg / m^2)	26.3 ± 4.6	24.9 ± 4.0
BP (mmHg)	122.8 ± 16.4 73.9 ± 11.2	117.8 ± 15.9 73.5 ±10.1

Table 6.1: Demographic and Clinical Characteristics of Study Participants. *T1DM: Type 1 Diabetes Mellitus; BMI: Body Mass Index; BP: blood Pressure*

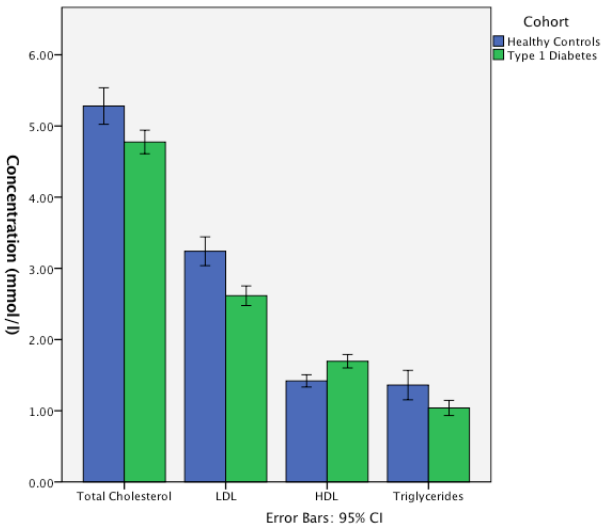

Figure 6.1: Baseline differences in the lipid profile. *LDL: Low-density lipoprotein; HDL: High-density lipoprotein; CI: Confidence Interval*

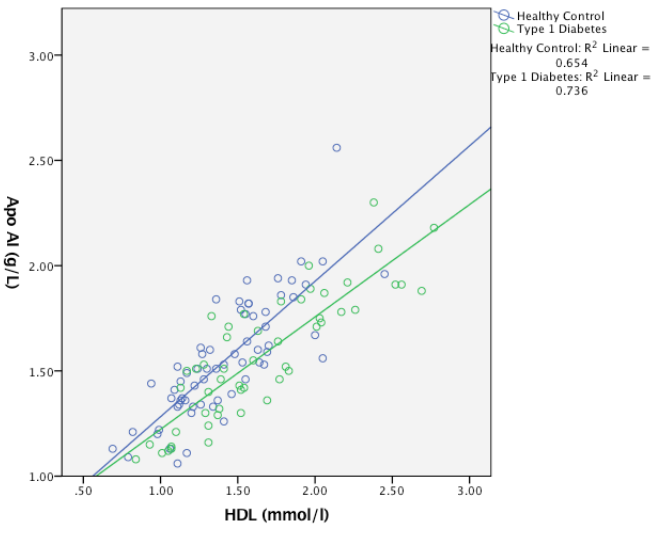

Figure 6.2: Correlation between HDL-cholesterol and Apo AI. *Apo AI: Apolipoprotein AI; HDL: High-density lipoprotein*

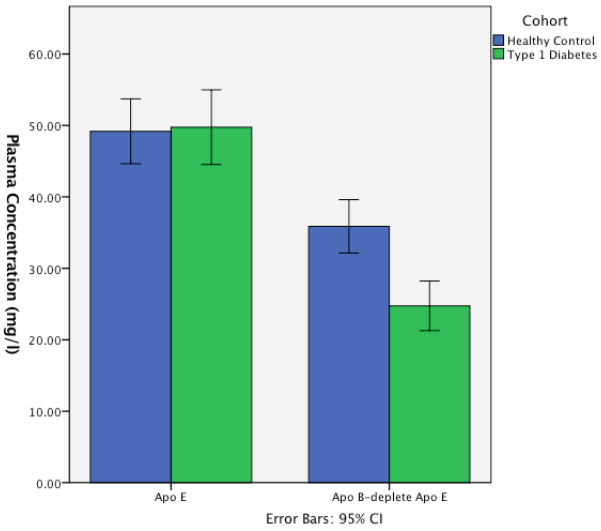

Figure 6.3: Apo E distribution. *Apo E: Apolipoprotein E; Apo B: Apolipoprotein B*

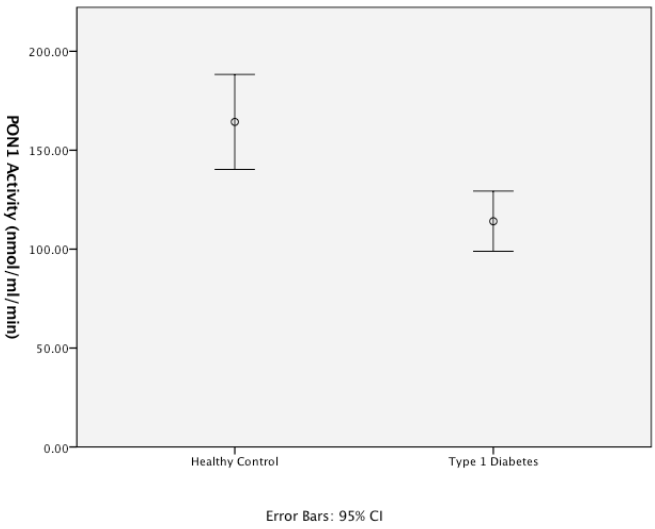

Figure 6.4: PON1 Activity in healthy adults and patients with Type 1 Diabetes. *PON1: Paraoxonase-1; CI: Confidence Interval*

	Healthy Controls (n = 104)	T1DM (n = 85)	T1DM with microalbuminuria (n = 6)
Neuropathy Symptom Profile (/38)	-	3.8 ± 5.1	13.5 ± 5.3**
Diabetic Neuropathy Symptom Score (/4)	0 ± 0	0.7 ± 1.2	3.0 ± 2.0*
Neuropathy Disability Score (/10)	-	2.6 ± 2.5	6.5 ± 1.9*
Vibration Perception Threshold (microns)	-	13.09 ± 8.60	31.94 ± 14.68**
QST – Cold Threshold (^oC)	-	23.24 ± 9.71	9.50 ± 8.75*
QST – Warm Threshold (^oC)	-	38.38 ± 9.40	47.58 ± 4.72
QST – Cold-induced Pain (^oC)	-	13.36 ± 9.56	2.68 ± 4.89
QST – Warm-induced Pain (^oC)	-	43.99 ± 8.47	49.30 ± 1.40
Corneal Nerve Fibre Density ($/mm^2$)	-	20.13 ± 8.61	14.73 ± 5.28
Corneal Nerve Branch Density ($/mm^2$)	-	23.82 ± 13.27	18.08 ± 3.81
Corneal Nerve Fibre Length (mm/mm^2)	-	12.76 ± 3.93	9.81 ± 2.04

Table 6.2: Assessments of neuropathy in the presence of microalbuminuria. *T1DM: Type 1 Diabetes Mellitus; QST: Quantitative Sensory Testing. * < 0.01; ** < 0.001*

	Healthy Controls (n = 104)	T1DM (n = 85)	T1DM with microalbuminuria (n = 6)
HDL Cholesterol (mmol/l)	1.42 ± 0.35	1.72 ± 0.45**	1.37 ± 0.29
Apo AI (g/L)	1.55 ± 0.28	1.58 ± 0.30	1.46 ± 0.27
CETP Activity (nmol/ml/h)	26.73 ± 2.95	26.30 ± 3.74	25.71 ± 3.66
Cholesterol Efflux Capacity (%)	16.62 ± 4.92	17.51 ± 4.55	16.86 ± 4.66
PON1 Activity (nmol/ml/min)	164.30 ± 97.65	115.72 ± 74.24**	98.58 ± 61.94
HDL-Associated Apo E (mg/l)	35.80 ± 14.96	25.05 ± 12.71**	21.95 ± 9.14
CAC Score	-	3.81 ± 11.21	12.0 ± 0.0
CIMT (cm)	-	0.061 ± 0.013	0.068 ± 0.012

Table 6.3: Assessments of HDL Functionality and macrovascular complications in the presence of microalbuminuria. *T1DM: Type 1 Diabetes Mellitus; HDL: High-density lipoprotein; Apo AI: Apolipoprotein AI; CETP: Cholesteryl ester transfer protein; PON1: Paraoxonase-1; CAC: Coronary Artery Calcification; CIMT: Carotid intima media thickness.* ** < 0.001

Appendix – Multivariate modelling

Parameter	Initial Analysis	Adjusted for Age	Adjusted for Statin use	Adjusted for LDL-C	Adjusted for HDL-C
Apo AI	NS	NS	NS	0.047	NS
Apo B	NS	0.025	NS	0.030	0.015
Glycated Apo B	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Apo B-deplete Apo E	< 0.001	< 0.001	< 0.001	0.004	NS
Apo CIII	NS	0.034	NS	NS	NS
Apo M	NS	NS	0.002	0.005	0.019
Glucose	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
HDL-C	< 0.001	< 0.001	< 0.001	< 0.001	NS
LDL-C	< 0.001	< 0.001	< 0.001	NS	NS
Oxidized LDL-C	NS	< 0.001	< 0.001	NS	NS
MPO Activity	0.001	0.018	0.011	0.004	0.001
PON1 Activity	< 0.001	0.002	0.002	0.024	0.05
Triglycerides	0.003	< 0.001	0.001	0.033	NS

Appendix Table 6.4: Effect of adjustments for age, statin use and the lipid profile on difference in measured parameters between healthy controls and patients with T1DM. No effect of T1DM was observed on total Apo E, cholesterol efflux capacity, CETP Activity, hs-CRP or SAA throughout the analysis. *LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; Apo AI : Apolipoprotein AI; Apo B: Apolipoprotein B: Apo E: Apolipoprotein E; Apo CIII: Apolipoprotein CIII; Apo M: Apolipoprotein M; MPO: Myeloperoxidase; PON1: Paraoxonase-1*

Appendix – Prediction of Subclinical Atherosclerosis

Ranked Correlation	Coronary Artery Calcification Score (*R*, *P*)	Carotid Intima Media Thickness (*R*, *P*)
1	Duration of Diabetes (0.38, 0.007)	Age (0.62, < 0.0001)
2	Age (0.37, 0.009)	Duration of Diabetes (0.48, < 0.001)
3	Systolic BP (0.34, 0.017)	LDL Cholesterol (0.41, 0.002)
4	PON1 Activity (-0.26, 0.053)	Glycated Apo B (0.40, 0.003)
5	SAA (0.25, 0.063)	Apo B (0.37, 0.006)

Appendix Table 6.5: Factors predicting subclinical atherosclerosis in T1DM. *BP: Blood Pressure; PON1: Paraoxonase-1; SAA: Serum Amyloid A; LDL: Low-density lipoprotein; Apo B: Apolipoprotein B*

Appendix – Prediction of Neuropathy

Ranked Correlation	Neuropathy Symptom Profile (*R*, *P*)	Neuropathy Disability Score (*R*, *P*)	Vibration Perception Threshold (*R*, *P*)	Corneal Nerve Fibre Density (*R*, *P*)	Corneal Nerve Branch Density (*R*, *P*)	Corneal Nerve Fibre Length (*R*, *P*)
1	Urinary Apo AI (0.64, < 0.0001)	Age (0.48, < 0.001)	Apo M (0.54, < 0.0001)	Duration of Diabetes (-0.40, 0.002)	HDL (-0.35, 0.007)	Duration of Diabetes (-0.42, < 0.001)
2	Urinary MCR (0.52, < 0.0001)	Apo M (0.47, < 0.001)	Urinary MCR (0.50, < 0.0001)	BMI (-0.30, 0.016)	Duration of Diabetes (-0.30, 0.014)	hsCRP (-0.35, 0.006)
3	Apo M (0.51, < 0.0001)	MPO Activity (-0.43, < 0.001)	Age (0.46, < 0.001)	HbA1c (-0.29, 0.021)	Apo CIII (-0.27, 0.026)	Diastolic BP (0.26, 0.035)
4	Triglycerides (0.51, < 0.0001)	VCAM-1 (0.40, 0.002)	Apo AI (-0.45, < 0.001)	hsCRP (-0.28, 0.023)	hsCRP (-0.27, 0.027)	Urinary Apo AI (-0.25, 0.037)
5	OxLDL (0.37, 0.004)	Apo AI (-0.37, 0.004)	VCAM-1 (0.41, 0.001)	Diastolic BP (0.27, 0.025)	Diastolic BP (0.25, 0.037)	OxLDL (-0.25, 0.037)

Appendix Table 6.6: Factors Predicting Diabetic Sensory Neuropathy in T1DM. *Apo Apolipoprotein AI; MCR: Microalbumin Creatinine Ratio; OxLDL: Oxidized LDL; MPO: Myeloperoxidase; VCAM-1: Vascular Cell Adhesion Molecule 1; BMI: Body Mass Index; HbA1c: Glycated Haemoglobin; hsCRP: High-sensitivity C-Reactive Protein; BP: Blood Pressure*

7. Assessing Cardiovascular Risk in Type 1 Diabetes Mellitus

Abstract

Introduction

Type 1 Diabetes Mellitus (T1DM) is associated with a significantly increased risk of atherosclerotic cardiovascular disease. Progress has been made through improved management of known risk factors but a significant residual relative risk remains.

Method

In this cross-sectional study of 91 patients with T1DM and 104 healthy volunteers we examine qualitative lipoprotein abnormalities and selected biomarkers for cardiovascular disease, and assess their value beyond current guidelines for the management of cardiovascular risk in T1DM.

Results

T1DM was associated with relative increases in apolipoprotein (apo) B, small-dense low-density lipoprotein (LDL), glycated apo B and oxidized LDL levels, with reduced apo AI concentrations and high-density lipoprotein functionality. Cystatin C, *N*-ε-carboxymethyl-lysine, lipoprotein-phospholipase A2 (lp-PLA2) and vascular cell adhesion molecule 1 concentrations were significantly increased in T1DM compared to healthy controls. Examination of the relationships between measured and calculated parameters revealed that age and duration of diabetes are most predictive of subclinical atherosclerosis assessed by coronary artery calcification scoring and carotid doppler studies.

Discussion

This study confirms the value of LDL-lowering in reducing cardiovascular risk in T1DM, demonstrating significantly elevated levels of atherogenic LDL in T1DM, associated with increased inflammation and endothelial dysfunction. We report potentially important alterations in Cystatin C, Leucine-rich α-2-glycoprotein 1 and lp-PLA2 which may be of value in improving cardiovascular risk assessment in T1DM.

Introduction

Diabetes mellitus is associated with a considerably increased risk of premature atherosclerosis [14, 15]. Early studies of cardiovascular mortality in type 1 diabetes (T1DM) suggested that risk only significantly increases after the development of nephropathy, which coincides with a marked deterioration of the lipid profile and blood pressure [165]. More recent analyses have suggested that improved management of other risk factors can reduce the overall relative risk to 3.0 for women and 2.3 for men [167]. Importantly this relative risk does not appear to be related to disease duration.

Although the correlation between diabetes and coronary heart disease (CHD) is well established, the underlying mechanisms remain poorly understood [291]. The risk of CHD is greater at any given level of serum cholesterol in patients with diabetes and its association with hypertriglyceridaemia is stronger than in the general population [181]. Importantly, there is strong and convincing evidence that cholesterol lowering is at least as effective in reducing CHD in patients with diabetes as in the general population [182-184].

In T1DM, hypertriglyceridaemia may occur, but high-density lipoprotein (HDL) cholesterol levels are often normal or even high unless glycaemic control is poor or nephropathy has developed [16]. The apparently normal serum cholesterol concentrations observed in T1DM led to the widespread erroneous belief that glycaemia alone might explain the observed high CHD rates. However, the relatively normal cholesterol levels hide an atherogenic lipid profile, with increased intermediate-density lipoprotein and small dense low-density lipoprotein (sdLDL), and dysfunctional HDL [207-209].

Improved glycaemic control generally has favourable effects on lipoprotein levels in diabetes, with a reduction in cholesterol and triglyceride levels through decreased circulating very-low-density lipoprotein (VLDL) and by increased catabolism of low-density lipoprotein (LDL) through reduced glycation and upregulation of LDL receptors [186, 187]. It is certainly possible that any cardiovascular benefit which might be derived from intensive glucose-lowering is related to effects on lipoprotein metabolism rather than directly through altered glycaemia [188]. In addition, patients with diabetes show qualitative and kinetic abnormalities for all lipoproteins [191].

In this study we explored these lipoprotein abnormalities, and selected biomarkers with the potential to predict cardiovascular disease, assessing their value beyond current recommendations for the management of patients with T1DM. We considered the effect of T1DM on circulating levels of these markers, and their association with subclinical atherosclerosis assessed through cardiac computed tomography (CT) and carotid doppler studies.

Method

Patients with T1DM were recruited through Central Manchester University Hospitals NHS Foundation Trust, University Hospital of South Manchester, Lancashire Teaching Hospitals NHS Foundation Trust, Bradford Institute for Health Research, and through the Diabetes Research Network 'Consent for Approach' Database (Help Diabeates®). The study was performed according to the Declaration of Helsinki and was approved by the National Research Ethics Service.

All participants were reviewed to assess basic demographic details, type and duration of diabetes, co-morbid conditions, known complications, insulin and lipid-lowering medication use, and the use of tobacco and alcohol. Patients with a history of coronary heart disease were excluded. Measurements were taken for height, weight and blood pressure, a fasting blood sample taken and urinalysis performed. Patients with T1DM were divided into 2 cohorts according to recruitment site: Cohort 1 had assessment of Coronary Artery Calcification by cardiac CT; Cohort 2 had assessment of carotid intima media thickness (CIMT) and atheroma burden by carotid doppler studies.

Healthy controls were recruited through the University of Manchester, Central Manchester University Hospitals NHS Foundation Trust and Barlow Medical Centre. Volunteers with any history suggestive of coronary heart disease, vascular insufficiency or diabetes, using any lipid lowering drugs (including omega fatty acid supplements) were excluded.

Demographic and clinical characteristics of the study participants are shown in Table 7.1.

Venous blood samples were collected after overnight fasting and serum and EDTA-plasma isolated by centrifugation at 2000 x G for 15 minutes at 4^{o}C within 2 hours of collection. Samples were aliquoted, anonymized and stored at 4^{o}C until all clinical laboratory testing was complete. Remaining samples were frozen to -80^{o}C.

Glycated haemoglobin (HbA1c) was analyzed by high-performance liquid chromatography on a VARIANT II TURBO Hemoglobin Testing System (Bio-Rad, Hercules, CA, USA). Glucose, cholesterol and triglycerides were determined by enzymatic hydrolysis and precipitation using glucose oxidase phenol 4 aminoantipyrine (GOD-PAP), cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) and glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) methods respectively (ABX Horiba-UK). HDL cholesterol was measured by a direct second-generation homogeneous method (Roche Diagnostics) and LDL cholesterol was estimated using the Friedewald Formula. Apolipoprotein AI (Apo AI) and Apolipoprotein B (Apo B) were measured

immunoturbidimetrically. A Cobas Mira autoanalyzer (ABX Horiba-UK, Northampton, UK) was used for all of these assays. Creatinine, cystatin C, high-sensitivity CRP (hsCRP) and sdLDL were measured immunoturbidimetrically using a Randox Daytona autoanalyzer (Randox, Co. Antrim, UK).

Apolipoprotein B48 (Apo B48) (MyBioSource.com, San Diego, CA, USA) and Glycated Apo B (Exocell, Philadelphia, PA, USA) were measured by indirect competitive ELISA. Apolipoprotein C-III (Apo CIII) (Sigma-Aldrich, St. Louis, MO, USA), Apolipoprotein M (Apo M) (Holzel Diagnostika Handels GmbH, Cologne, Germany), Intercellular Adhesion Molecule 1 (ICAM-1) (R&D Systems Europe, Abingdon, UK), Interleukin 6 (IL-6) (R&D Systems Europe, Abingdon, UK), Leucine-rich α-2 glycoprotein (LRG) (Immuno-Biological Laboratories, Minneapolis, MN, USA), Lipoprotein (a) (Lp(a)) (Mercodia, Uppsala, Sweden), Lipoprotein-associated phospholipase A2 (Lp-PLA2) (Uscn Life Science Inc., Buckingham, UK), Myeloperoxidase (MPO) mass (R&D Systems Europe, Abingdon, UK), *N*-ε-carboxymethyl-lysine (CML) (MyBioSource Inc., USA), Oxidized LDL (Mercodia AB, Uppsala, Sweden), Proprotein convertase subtilisin / kexin type 9 (PCSK9) (R&D Systems Europe, Abingdon, UK), Serum Amyloid A (SAA) (ThermoFisher Scientific, Loughborough, UK), Tumour Necrosis Factor alpha (TNF-α) (R&D Systems Europe, Abingdon, UK), Vascular Cell Adhesion Molecule 1 (VCAM-1) (R&D Systems Europe, Abingdon, UK) and 3-Nitrotyrosine (3-NT) (MyBioSource Inc., San Diego, CA, USA) were assessed by quantitative sandwich ELISA. The presence of microalbuminuria was assessed by measurement of albumin-to-creatinine ratio in a random spot urine collection [437].

Cholesterol efflux capacity was assessed in a method based upon that of Khera *et al* [76]. Paraoxonase-1 (PON1) was determined by a semi-automated micro-titre plate method using paraoxon (*O,O*-Diethyl *O*-(4-nitrophenyl)phosphate) as a substrate. MPO activity was assessed using a colourimetric activity assay kit (Sigma-Aldrich, St. Louis, MO, USA).

40 patients with T1DM underwent Coronary Artery Calcification (CAC) Score determination by electron-beam CT using an automated program based on the Agatson method as a screening tool to identify subclinical atherosclerosis [27]. A CAC Score >10 was considered significant [28]. The carotid arteries were imaged in the remaining patients with T1DM with a Siemens Sequoia ultrasonography system (Siemens Medical Solutions, Mountain View, CA) with an 8- to 15-MHz linear array transducer. Examination included measurement of common and internal carotid artery flow velocities and CIMT at each of 3 scan planes. Here a mean CIMT of 0.06 cm was considered significant [29].

The clinical and lipid data were merged and clinical baseline characteristics and lipid profile-related parameters compared between groups by univariate and multivariate linear regression models to control for baseline differences between groups. Normal distribution

was tested for all data with the Kolmogorov-Smirnov, D'Agostino and Pearson omnibus and Shapiro-Wilk normality tests. A Bonferroni correction to account for multiple testing in multivariate model building was applied, with a significance level of 0.05, which corresponds to a p-value of 0.004. All analyses were performed with SPSS statistical software, version 19.0 (IBM, Zurich, Switzerland). Patients with T1DM were subdivided according to the presence of microalbuminuria. Significance was assessed with Student unpaired, two-tailed t tests. Simple linear correlations were calculated by determining the Pearson correlation coefficient *R*. Non-linear regression analysis was performed comparing biomarkers with CAC score and CIMT.

Results

Effects on the Lipid Profile

By definition, patients with T1DM had a higher fasting glucose (9.19 ± 4.41 *vs.* 4.50 ± 0.66 mmol/l, $P < 0.0001$). Consistent with previous reports, the routine lipid profile appeared to be less adverse in patients with T1DM (Figure 7.1), with both total and LDL cholesterol significantly lower than in healthy volunteers (4.78 ± 0.80 *vs.* 5.28 ± 1.05 mmol/l, $P = 0.0008$ and 2.62 ± 0.66 *vs.* 3.24 ± 0.82 mmol/l, $P < 0.0001$ respectively), and HDL cholesterol significantly higher (1.70 ± 0.45 *vs.* 1.42 ± 0.35 mmol/l, $P < 0.0001$). Triglycerides were also significantly lower in patients with T1DM (1.04 ± 0.51 *vs.* 1.36 ± 0.84 mmol/l, $P = 0.003$).

These apparently beneficial differences in the lipid profile persisted when patients with T1DM taking lipid-lowering medications (n = 16) were excluded from the analysis with total cholesterol, LDL cholesterol and triglycerides significantly lower ($P = 0.01$, $P < 0.0001$, and $P = 0.001$ respectively) and HDL cholesterol significantly higher ($P < 0.0001$) in patients with T1DM compared to healthy controls.

Although HDL cholesterol was significantly higher in patients with T1DM compared to healthy controls, no difference was observed in apo AI levels. When healthy controls were matched according to HDL cholesterol, apo AI levels were significantly lower in patients with T1DM.

Although mean LDL cholesterol was significantly higher in healthy volunteers than patients with T1DM ($P < 0.0001$), there was no difference in apo B levels. In contrast, both non-HDL cholesterol and estimated remnant cholesterol were significantly higher in healthy volunteers than in patients with T1DM ($P < 0.0001$ and 0.002 respectively). Adjustment for LDL cholesterol levels resulted in significantly higher apo B concentrations among patients with T1DM (0.80 ± 0.16 *vs.* 0.74 ± 0.09 g/L, $P = 0.04$) compared with healthy controls, but

no significant difference was observed between the two groups for other putative estimates of cardiovascular risk derived from the lipid profile (Table 7.2).

Again consistent with previous reports, these effects on HDL cholesterol and triglycerides were lost with the development of microalbuminuria (≥ 3.5 mg/mmol in females or ≥ 2.5 mg/mmol in males) with no significant difference in either parameter between healthy controls and patients with T1DM and microalbuminuria.

Atherogenic Modifications of LDL

The proportion of LDL having undergone atherogenic modification was significantly higher in patients with T1DM compared to healthy controls, with glycated apo B accounting for 3.84 ± 0.77% of the total apo B in T1DM *vs.* 2.30 ± 1.57% in healthy controls, $P < 0.0001$, and the proportion of oxidized LDL also significantly higher in T1DM at 15.66 ± 3.22 compared to 11.71 ± 5.64 μg/mmol in healthy controls, $P < 0.0001$. The correlation between glycated apo B and apo B, and oxidized LDL and LDL, was much stronger in patients with T1DM compared to healthy controls ($R = 0.47$, $P < 0.001$ *vs.* $R = 0.05$, P = NS and $R = 0.73$, $P < 0.0001$ *vs.* $R = -0.24$, $P = 0.026$ respectively). Small-dense LDL concentrations were also significantly higher in patients with T1DM compared to healthy controls following adjustment for LDL cholesterol (19.19 ± 10.05 *vs.* 12.83 ±8.60 mg/dl, $P = 0.005$) (Figure 7.2).

Glycated apo B and oxidized LDL levels showed a strong correlation with sdLDL ($R = 0.51$, $P < 0.0001$ and $R = 0.85$, $P < 0.0001$ respectively) in patients with T1DM, but not in healthy volunteers (Figure 7.3).

HDL Functionality

We have considered the effect of T1DM on HDL functionality elsewhere. Apo AI appeared to offer more protection against both glycation and oxidation in healthy controls compared to patients with T1DM ($R = -0.60$, $P < 0.0001$ *vs.* $R = -0.39$, $P = 0.003$ and $R = -0.26$, $P = 0.017$ *vs.* $R = 0.12$, P = NS respectively). Apo AI levels were also negatively correlated with markers of inflammation including hsCRP ($R = -0.29$, $P = 0.009$) in healthy controls, but not in patients with T1DM.

In addition to these effects of apo AI, significant reductions in PON1 activity (118.67 ± 73.79 *vs.* 164.30 ± 97.65 nmol/ml/min, $P = 0.0003$) and cholesterol efflux capacity (10.31 ± 3.00 *vs.* 12.54 ± 5.35%, $P = 0.02$) were also observed.

Candidate Biomarkers

Apo B48 levels were higher in patients with T1DM compared to healthy volunteers across all adjustments, although this difference did not reach statistical significance (Figure 7.4). In

contrast, apo CIII levels were non-significantly higher in healthy volunteers than in patients with T1DM. There was no significant difference in apo E levels between healthy volunteers and patients with T1DM, but the distribution of apo E was altered, with significantly more apo E associated with HDL in healthy controls compared with patients with T1DM (35.80 ± 14.96 *vs*. 24.74 ± 12.36 mg/l). Apo M levels were also higher in healthy volunteers than in patients with T1DM, although this difference only reached significance when patients taking lipid-lowering medications were excluded from the analysis (*P* = 0.002).

There was no significant difference in Lp(a) or total PCSK9 levels between healthy volunteers and patients with T1DM (Figure 7.5). *N*-ε-carboxymethyl-lysine (CML) levels were significantly higher in patients with T1DM than healthy controls (3.95 ± 3.72 *vs*. 2.88 ± 1.68 μmol/mol, *P* = 0.039). No significant difference in 3-nitrotyrosine levels was observed between healthy volunteers and patients with T1DM. Lipoprotein-associated phospholipase A2 levels were significantly higher in patients with T1DM compared to healthy volunteers following adjustments for lipid-lowering medications or LDL cholesterol levels (*P* = 0.021 and 0.008 respectively). MPO activity and mass were significantly higher in healthy controls compared to patients with T1DM (7.50 ± 5.77 *vs*. 4.63 ± 2.14 mU, *P* = 0.001, and 755.78 ± 506.808 *vs*. 449.63 ± 197.89 pmol/l, *P* < 0.0001, respectively).

Leucine-rich α-2-glycoprotein 1 concentrations were non-significantly higher (except in women, *P* = 0.016) in patients with T1DM compared to healthy controls. Even after adjustment for serum creatinine, cystatin C was significantly higher in patients with T1DM (0.85 ± 0.25 *vs*. 0.76 ± 0.12 mg/L, *P* = 0.003) than in healthy volunteers.

Levels of hsCRP and ICAM-1 were non-significantly higher, and levels of IL-6 and SAA non-significantly lower in patients with T1DM compared to healthy volunteers across all analyses. No significant difference in TNF-α levels was observed between healthy volunteers and patients with T1DM. Plasma VCAM-1 concentrations were significantly higher in patients with T1DM compared to healthy volunteers (440.61 ± 115.75 *vs*. 325.98 ± 42.59 ng/ml, *P* < 0.0001).

Prediction of subclinical atherosclerosis

The strongest relationships with CAC score were seen for duration of diabetes (*R* = 0.38, *P* = 0.007), age (*R* = 0.37, *P* = 0.009), systolic blood pressure (*R* = 0.34, *P* = 0.017), and PON1 activity (*R* = -0.26, *P* = 0.05). The strongest relationships with CIMT were seen for age (*R* = 0.62, *P* < 0.0001), duration of diabetes (*R* = 0.48, *P* < 0.001), LDL cholesterol (*R* = 0.41, *P* = 0.001), glycated apo B (*R* = 0.40, *P* = 0.002), apo B (*R* = 0.37, *P* = 0.004), non-HDL cholesterol (*R* = 0.34, *P* = 0.007), oxidized LDL (*R* = 0.33, *P* = 0.008), cystatin C (*R* = 0.33, *P* = 0.009), CML (*R* = 0.31, *P* = 0.014), and total cholesterol (*R* = 0.30, *P* = 0.016).

Post hoc analysis of the relationships between measured parameters and imaged coronary artery calcification or carotid intima media thickness suggested that the drivers of atherosclerosis may vary according to gender (Table 7.3).

Discussion

Cardiovascular Risk in Type 1 Diabetes Mellitus

Hyperglycaemia is well established as an independent risk factor for cardiovascular disease; this risk can be reduced in patients with T1DM if strict glycaemic control can be maintained [482]. Although more recent analyses have suggested a less marked effect, most authorities consider T1DM to confer at least a twofold excess risk, independently from other conventional risk factors [162, 163]. This study reports qualitative abnormalities in lipoproteins and other proteins implicated in the development of atherosclerosis.

A concerning feature of the data presented here is the low rate of achievement of glycaemic control targets, with a mean HbA1c 65 mmol/mol and just 6 study participants achieving the target HbA1c of 48 mmol/mol recommended by the National Institute for Health and Care Excellence (NICE) [483]. This cohort therefore will include patients at significantly increased risk of CHD. While features such as the presence of nephropathy or retinopathy identify higher risk groups, the use of other biomarkers of risk and likely need for enhanced treatment is often not appreciated.

Strategies to reduce the complications of diabetes are working but cardiovascular risk remains unacceptably high for patients with T1DM [167]. The efficacy of lowering LDL cholesterol in reducing cardiovascular morbidity and mortality is well established [91]. 16 patients in this study were receiving lipid-lowering medications. NICE recently updated its guidance and now advises clinicians to offer statin treatment for primary prevention to adults with T1DM who are over 40 years, have had diabetes for more than 10 years, or have established nephropathy or other cardiovascular risk factors [266]. If this guidance was followed a further 66 study participants would be offered statin treatment.

The relative risk is greater for women than men, and this study reports interesting qualitative lipoprotein differences according to gender. Previous work suggests that the excess relative risk in women is not explained by adverse changes in known cardiovascular risk factors [484].

Effects on the Lipid Profile

The revised American Diabetes Association treatment guidelines on lipid management still suggest LDL cholesterol ≥ 2.6 mmol/l as a marker of increased cardiovascular risk [265]. 48 patients in this study had LDL cholesterol above this threshold. In risk prediction models for

patients with T1DM, total cholesterol and HDL cholesterol are more important than LDL cholesterol in predicting adverse cardiovascular outcomes [485].

Strong correlations between apo B and non-HDL cholesterol have been reported in T1DM [486]. Non-HDL cholesterol was established to improve risk estimation beyond LDL cholesterol from Friedewald's formula but in a cohort such as this without significant hypertriglyceridaemia apo B measurement may provide a more complete picture of the lipoprotein profile as it will account for small, dense and more atherogenic particles [487]. It is significant that apo B levels are significantly higher in patients with T1DM than in healthy volunteers when other putative estimates of cardiovascular risk did not recognise any increased risk associated with T1DM.

In a recent large observational study cardiovascular risk in T1DM was predicted by total cholesterol / HDL cholesterol, and non-HDL cholesterol but not LDL cholesterol [488]. In the INTERHEART study the non-fasting apo B / apo AI ratio was a better predictor of myocardial infarction than any single lipid or apolipoprotein concentration, or any combination or ratio of measurements [489]. Another recent observational study found apo B / apo AI to be the best predictor of CHD for women with normoalbuminuria and acceptable glycaemic control, and atherogenic apo B to be the best predictor in patients with macroalbuminuria [490]. Clearly these measures are only of use in identifying patients with T1DM at higher risk, and may prove falsely reassuring if compared to values from people without diabetes.

The lipoprotein changes with nephropathy described here are consistent with those demonstrated in the Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications cohort [19]. The small number of patients with microalbuminuria included in this study relates to angiotensin-converting enzyme inhibitor use [491].

Atherogenic Modifications of Low-density Lipoprotein Cholesterol

Atherosclerosis progression is associated with high levels of LDL and, more particularly, sdLDL [315]. Small-dense LDL penetrates the arterial wall more easily than large buoyant LDL, is less resistant to oxidative stress, has a prolonged plasma half-life and has a reduced binding affinity for LDL receptors [492]. These characteristics of sdLDL would all be expected to contribute to increased atherogenicity, which may also be partly linked to non-oxidative modifications of apo B [317]. Small-dense LDL is more readily glycated than larger more buoyant LDL both *in vivo* and *in vitro,* possibly because a higher proportion of the apo B molecule is exposed to glucose [318].

The role of glucose (or species derived from glucose) in inducing atherogenic LDL modifications is of particular relevance to diabetes-associated atherosclerosis [292]. There

is considerable evidence implicating lipid peroxidation and oxidative modification of LDL in atherosclerotic lesion development [324], but non-enzymatic glycation of LDL may be just as important. Glycated LDL may be more susceptible to oxidation or itself represent an atherogenic modification [207, 208]. Glycated LDL is present in the circulation under physiological conditions and at higher concentrations in diabetes and in people destined to experience myocardial infarction [205]. LDL glycation might ultimately prove at least as important in atherogenesis as oxidation [71], and the significantly increased levels of LDL glycation and oxidation observed in T1DM in this study are an important finding. Interestingly, statins also reduce circulating concentrations, likely by reducing LDL available to undergo glycation [205]. We previously reported that HDL impedes the glycation of LDL [71].

I have also employed machine learning random forest analyses (Figure 7.6) to explore the relative importance of factors correlating with glycated apo B levels. This method suggests that fasting glucose, oxidized LDL and sdLDL are most predictive of the glycated apo B concentration. This algorithm also suggests that these predictors may be co-linear, with independent associations. This data supports the observation that LDL-lowering is at least as important to reducing diabetic macrovascular complications as glucose-lowering. The parameters included here account for more than 73% (95% CI 0.585 – 0.843) of the variability in glycated apo B (Figure 7.7). Further work is warranted to discover the contribution of other factors to glycated apo B levels, as these factors may also be amenable to therapeutic interventions.

High-density Lipoprotein Functionality

In patients with T1DM with good glycaemic control, insulin upregulates lipoprotein lipase, increasing the production of small HDL particles [210, 211], frequently to higher than normal HDL cholesterol levels [212]. HDL cholesterol in diabetes has thus not arisen physiologically; HDL dysfunction is suggested by the observation that these high HDL cholesterol levels do not appear to offer protection against CHD [11, 130]. The reduced anti-oxidative and anti-inflammatory effects of HDL described here are consistent with previous reports in patients with diabetes, alongside an impaired cholesterol efflux capacity and ability to counteract the inhibition of endothelium-dependent vasorelaxation by oxidized LDL [192, 213].

We have considered altered HDL functionality in T1DM elsewhere, but it is increasingly implicated in the residual cardiovascular risk associated with T1DM. There is a significant body of evidence to support a role for HDL-associated PON1 in atherosclerosis, and PON1 activity in serum is inversely related to the risk of cardiovascular disease [390]. Under pathological conditions such as T1DM, PON1 dissociates from HDL to the lipoprotein-free serum fraction, where it is less biologically active [458]. Dysfunctional HDL associated with

lower PON1 activity has previously been linked to increased endothelial VCAM-1 expressions and reduced NO production by an oxidized LDL receptor (LOX-1) dependent pathway [467].

Candidate Biomarkers of Cardiovascular Risk

As described above, both apo AI and apo B levels appear unaffected by the presence of T1DM, but apo AI is significantly lower (P = 0.013) in T1DM when controls are matched according to HDL cholesterol and apo B is significantly higher (P = 0.042) in T1DM when controls are matched according to LDL cholesterol. These findings represent important additional cardiovascular risk not evident from the standard lipid profile. Although not quite reaching statistical significance, the higher levels of apo B48 observed in patients with T1DM merit further attention, and might reasonably be expected to be significant post-prandially. Apo B48 is present in chylomicrons and has been suggested as a biomarker for postprandial changes in lipoprotein distribution and associated cardiovascular risk. Both apo B and apo CIII-containing lipoproteins have been linked with nephropathy and atherosclerosis, and these effects may be enhanced in T1DM [493]. Here significantly more apo CIII was associated with HDL cholesterol following the development of microalbuminuria, where it is considered pro-inflammatory and indicative of increased cardiovascular risk [478].

Elevated Lp(a) is an independent causal risk factor for atherosclerotic cardiovascular disease [494]. Intensive treatment in the DCCT was associated with decreased Lp(a) as well as apo B [495]. The lack of effect of T1DM on Lp(a) levels is consistent with previous studies and its status as a genetically determined cardiovascular risk factor [496]. A previous observational study suggested an Lp(a) value > 30 mg/dl might be of predictive value in the assessment of cardiovascular risk in T1DM [497]; this approach would have identified 7 patients with T1DM who might benefit from more aggressive management of their cardiovascular risk.

To our knowledge this is the first report of PCSK9 levels in T1DM. The lack of effect of T1DM on PCSK9 activity suggests that patients with T1DM might draw as much benefit from LDL-lowering with PCSK9 inhibitors as the general population.

Hyperglycaemia induces the non-enzymatic glycation of proteins, resulting in the formation of advanced glycation end products (AGEs), including the predominant CML, which interact with the arterial wall through specific receptors, including receptors for AGE (RAGE), contributing to atherosclerosis [293]. The finding of increased CML in patients with T1DM is consistent with previous investigations of AGEs in T1DM [498].

The changes in LDL particle size and its glycation and oxidation described above are associated with endothelial dysfunction and CHD [499]. Endothelial injury and dysfunction

are a common link for all cardiovascular risk factors [500]. Activation of oxidative stress by hyperglycaemia plays a major role in the pathogenesis of complications in diabetes [322]. The inhibitory effect of oxidized LDL on endothelium-dependent vasorelaxation is mainly related to decreased bioavailability of nitric oxide [501]. Superoxide reacts with nitric oxide causing a nitrosative stress with generation of metabolic dervivatives including peroxynitrite and nitrotyrosine [502]. The toxicity of these substances can cause endothelial damage and thus complications [323]. The lack of effect of T1DM on 3-NT levels is therefore an unexpected finding and warrants further investigation.

Lp-PLA2 has been shown to be a cardiovascular risk marker independent of and additive to traditional risk factors [503]. Most Lp-PLA2 is associated with LDL so the significantly higher concentrations observed here following adjustment for LDL cholesterol are an important finding [23]. Lp-PLA2 specifically hydrolyzes oxidized phospholipids on oxidized LDL particles within the arterial intima, stimulating the expression of endothelial adhesion molecules and cytokines [504]. In a prospective population-based study subjects with high Lp-PLA2 activity and high concentrations of oxidized phospholipids on apo B particles were reported to have nearly double the risk of future cardiovascular events compared to subjects with high levels of one of these factors [505]. Here we have demonstrated for the first time that patients with T1DM have elevated Lp-PLA2 activity in association with significantly increased oxidized LDL. In the Atherosclerosis Risk In Communities study, individuals who had increased levels of both Lp-PLA2 and hsCRP were 3 times more likely to have a coronary event compared with individuals with low levels of Lp-PLA2 and hsCRP [506].

The observation of significantly lower MPO mass and activity in patients with T1DM reported here may seem surprising, but other studies have also reported significantly lower MPO levels in patients with T1DM when compared to healthy volunteers [464]. Anti-MPO antibodies have been detected in patients with T1DM, associated with a state of chronic neutrophil activation [465], which would be expected to increase vascular inflammation.

Leucine-rich α-2 glycoprotein was recently identified as a predictor of endothelial dysfunction and peripheral arterial disease in type 2 diabetes [22], but this is the first report of altered levels in T1DM. Interestingly, women were also found to have higher levels in the previous study. The potential significance of these gender differences and the value of leucine-rich α-2 glycoprotein as a biomarker warrant further investigation; it is not yet clear whether elevated levels represent microvascular or macrovascular responses.

Cystatin C is well established in the assessment of renal function and nephropathy in T1DM, but recent interest has centred on its potential value in cardiovascular risk assessment [507]. Epidemiological studies show a strong association between circulating cystatin C and

the development of CHD independent of renal function [508]. The higher levels of cystatin C demonstrated here in T1DM after adjustment for serum creatinine may therefore be relevant to the development of CHD in T1DM, and thus merit further investigation.

Atherosclerosis is a systemic, low-grade inflammatory disease [500]. Biomarkers of inflammation may aid in predicting cardiovascular risk. An increase in inflammatory cytokines contributes to plaque instability in patients with T1DM [509]. The accumulation of inflammatory cytokines is believed to cause endothelial injury and altered coagulation, resulting in increased cardiovascular risk [509]. Elevated hsCRP may reflect systemic inflammation but may also directly contribute to atherosclerosis [510]. In a large meta-analysis of prospective studies CRP was strongly associated with the risk of CHD and ischaemic stroke in people without any history of vascular disease [511]. Statins decrease systemic CRP levels, and the use of such medication could have altered CRP levels and the inherent cardiovascular risk in this study [46]. SAA is a family of acute-phase proteins which may also contribute to CHD development [512]. It is primarily carried in HDL, but is also associated with apo B-containing lipoproteins [12]. The significant increase in VCAM-1 levels compared to other inflammatory markers described here in patients with T1DM is of interest given recent reports of increased VCAM-1 expression in human endothelial cells on exposure to glycated LDL [513].

Prediction of Subclinical Atherosclerosis

CIMT is a good surrogate marker of atherosclerosis and an independent predictor of cardiovascular events [449]. Consistent with their increased risk for cardiovascular disease, CIMT is increased in people with T1DM [514]. Severity of atherosclerosis in T1DM as measured by carotid ultrasound has been correlated with LDL subfractions, LDL particle number, LDL cholesterol, apo B, age, hypertension, smoking, retinopathy, and HDL cholesterol [515]. CIMT progression in the Epidemiology of Diabetes Interventions and Complications trial was associated with age, systolic BP, smoking, the LDL / HDL ratio, urinary albumin excretion rate and HbA1c [516]. Increased CIMT has also been associated with insulin resistance [517]. Small-dense LDL has also been shown to be closely associated with CIMT, and more so than other lipid parameters, including LDL, apo B, total cholesterol, HDL, and apo AI levels [518].

Coronary artery calcium is another well established index of atherosclerosis [519]. Patients with T1DM with both elevated apo B and non-HDL cholesterol were recently shown to have greater coronary artery calcification compared not only with patients with normal values but also with patients with elevated apo B alone, suggesting that apo B and non-HDL cholesterol might be viewed as complementary rather than competitive indices of cardiovascular risk in T1DM [486]. In an analysis of the Pittsburgh Epidemiology of Diabetes Complications Study baseline HbA1c, duration of diabetes, increased albumin excretion,

lower insulin doses, reduced renal function, higher diastolic BP and lipid profile were all predictive of CHD [520]. Progression of CAC was strongly associated with HbA1c [521]. Progression of coronary calcification is also positively correlated with non-HDL cholesterol [522].

In this study we have considered qualitative lipoprotein abnormalities and potential biomarkers for the increased risk of CHD associated with T1DM. We have confirmed or refuted associations with a number of these, showing a variable relationship with subclinical atherosclerosis according to gender, while confirming the importance of LDL-related parameters to cardiovascular risk, by showing that some markers only gain prominence once LDL-lowering has been achieved.

A weakness in the present study is its cross-sectional, observational approach, and it is therefore difficult to draw causal relationships. Other study limitations include the small sample size, especially in the microalbuminuria group.

Future work will utilize machine learning to further explore the relationships between biomarkers of cardiovascular risk in T1DM, alongside analysis of other novel markers such as sphingolipids and ceramides.

	Patients with T1DM (n = 91)	Healthy Controls (n = 104)
Male / Female (%)	42 (46.1) / 49 (53.8)	44 (42.3) / 60 (57.7)
Age (years)	41.2 ± 13.5	36.8 ± 10.6
Duration of Diabetes (years)	20.8 ± 12.5	-
BMI (kg / m^2)	26.3 ± 4.6	24.9 ± 4.0
BP (mmHg)	122.8 ± 16.4 / 73.9 ± 11.2	117.8 ± 15.9 / 73.5 ±10.1

Table 7.1: Demographic and Clinical Characteristics of Study Participants. *T1DM: Type 1 Diabetes Mellitus; BMI: Body Mass Index; BP: blood Pressure*

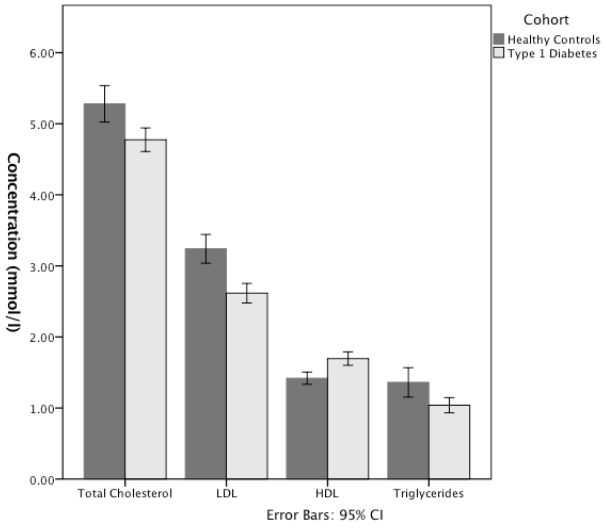

Figure 7.1: Effects of Type 1 Diabetes Mellitus on the Lipid Profile. *LDL: Low-density lipoprotein; HDL: High-density lipoprotein; CI: Confidence Interval*

	T1DM	Healthy Controls matched for LDL Cholesterol	T1DM + Microalbuminuria	Healthy Controls matched for LDL Cholesterol
LDL Cholesterol (mmol/l)	2.62 ± 0.66	2.61 ± 0.51	2.30 ± 0.81	2.29 ± 0.45
Non-HDL Cholesterol (mmol/l)	3.08 ± 0.71	3.17 ± 0.65	2.94 ± 0.89	2.83 ± 0.55
Apo B (g/L)	0.80 ± 0.16	0.74 ± 0.09*	0.76 ± 0.22	0.70 ± 0.11
Remnant Cholesterol (mmol/l)	0.46 ± 0.24	0.57 ± 0.40	0.64 ± 0.28	0.54 ± 0.36
Total / HDL Cholesterol	2.96 ± 0.72	3.57 ± 1.00**	3.28 ± 0.97	3.29 ± 1.04
Apo B / Apo AI	0.53 ± 0.13	0.53 ± 0.10	0.52 ± 0.11	0.51 ± 0.12

Table 7.2: Lipid profile markers and derived estimates of cardiovascular risk in patients with T1DM and healthy controls matched for LDL cholesterol levels. *LDL: Low-density lipoprotein; T1DM: Type 1 Diabetes Mellitus; HDL: High-density lipoprotein; Apo B: Apolipoprotein B; Apo AI: Apolipoprotein AI.* * $P < 0.05$; ** $P < 0.001$; † $P < 0.05$

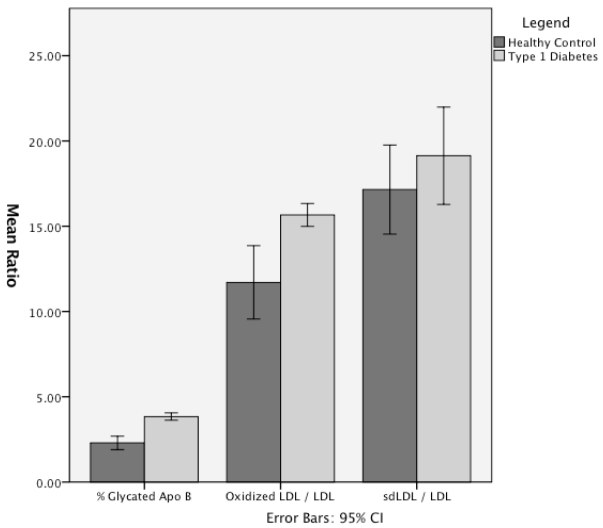

Figure 7.2: Proportion of LDL Undergoing atherogenic modification. *Apo B: Apolipoprotein B; LDL: Low-density lipoprotein; sdLDL: Small-dense LDL; CI: Confidence Interval*

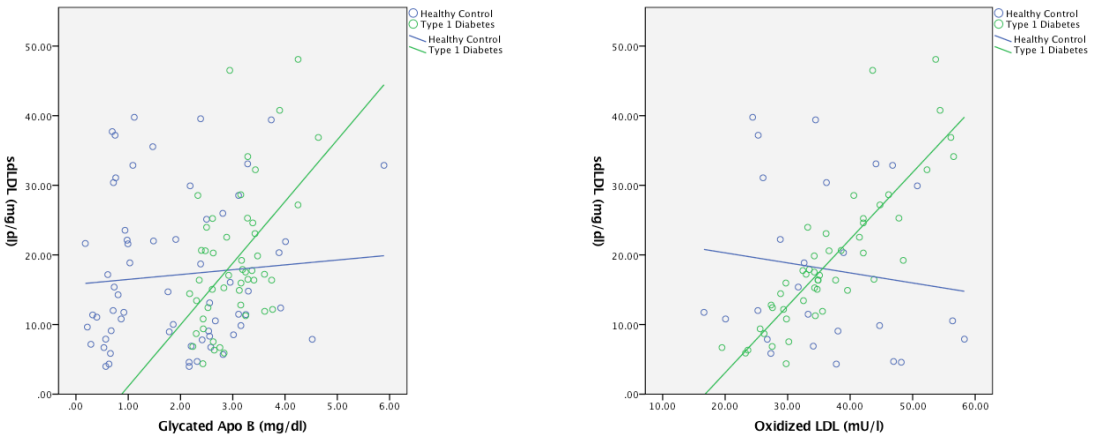

Figure 7.3: Relationship between Glycated Apo B, Oxidized LDL and sdLDL. *Apo B: Apolipoprotein B; LDL: Low-density lipoprotein; sdLDL: Small-dense LDL*

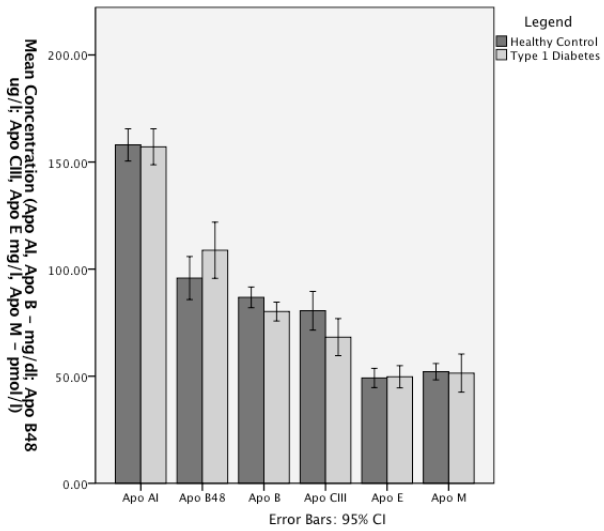

Figure 7.4: Apolipoprotein Levels in T1DM. *Apo: Apolipoprotein*

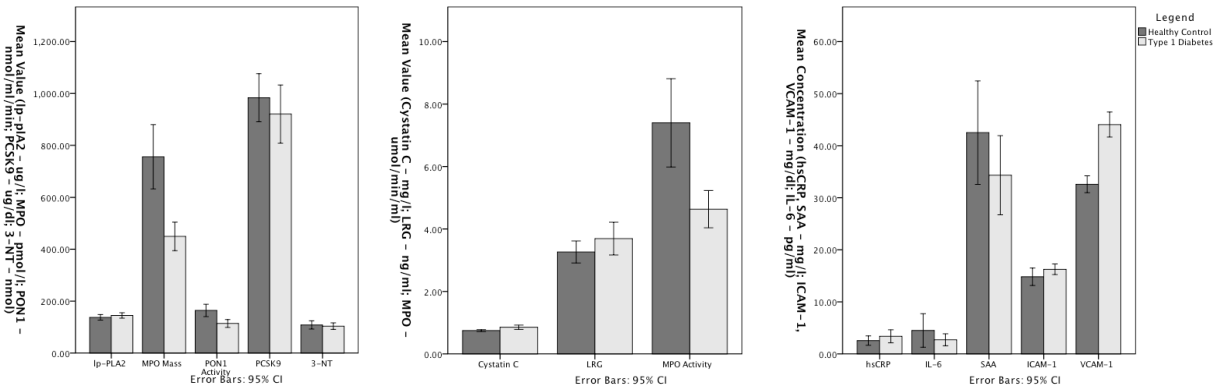

Figure 7.5: Candidate Biomarkers of Cardiovascular Risk in T1DM. *Lipoprotein-associated phospholipase A2; MPO: Myeloperoxidase; PON1: Paraoxonase-1; PCSK9: Proprotein convertase subtilisn/kexin type 9; 3-NT: 3-Nitrotyrosine; LRG: Leucine-rich α-2-glycoprotein 1; hsCRP: high-sensitivity C-reactive Protein; IL-6: Interleukin 6; SAA: Serum Amyloid A; ICAM-1: Intercellular Adhesion Molecule 1; VCAM-1: Vascular Cell Adhesion Molecule 1*

Rank	Males (No statin)	Males (statin)	Males (All)	Females (No statin)	Females (statin)	Females (All)
1	Age	MCR	Age	Age	Glucose	Age
2	Systolic BP	Glycated Apo B	Systolic BP	Duration of Diabetes	Waist circumference	Duration of Diabetes
3	Duration of Diabetes	SAA	Duration of Diabetes	Apo B	ICAM-1	LDL cholesterol
4	CML	Apo M	CML	sdLDL	PON1 Activity	Apo B
5	SAA	VCAM-1	Total / HDL cholesterol	Glucose	BMI	Non-HDL cholesterol
6	Total / HDL cholesterol	ICAM-1	HbA1c	LDL cholesterol	LDL	Oxidized LDL
7	HbA1c	Age	Waist circumference	Non-HDL cholesterol	Apo B	Total cholesterol
8	Waist circumference	Cystatin C	Apo B / Apo AI	Diastolic BP	Duration of Diabetes	Cystatin C
9	Diastolic BP	MPO Mass	Glycated Apo B	Oxidized LDL	Oxidized LDL	Glycated Apo B
10	Glycated Apo E	Waist circumference	Glycated Apo E	HbA1c	Cholesterol Efflux Capacity	ICAM-1

Table 7.3: Ranked correlations between measured parameters and imaged coronary artery calcification or carotid intima media thickness. *BP: Blood Pressure; CML: N-ε-carboxymethyl-lysine; SAA: Serum Amyloid A; HbA1c: Glycated haemoglobin; Apo: Apolipoprotein; HDL: High-density lipoprotein; MCR: Microalbumin Creatinine Ratio; VCAM-1: Vascular Cell Adhesion Molecule 1; ICAM-1: Intercellular Adhesion Molecule 1; MPO: Myeloperoxidase; LDL: Low-density lipoprotein; sdLDL: Small-dense LDL; PON1: Paraoxonase-1; BMI: Body Mass Index*

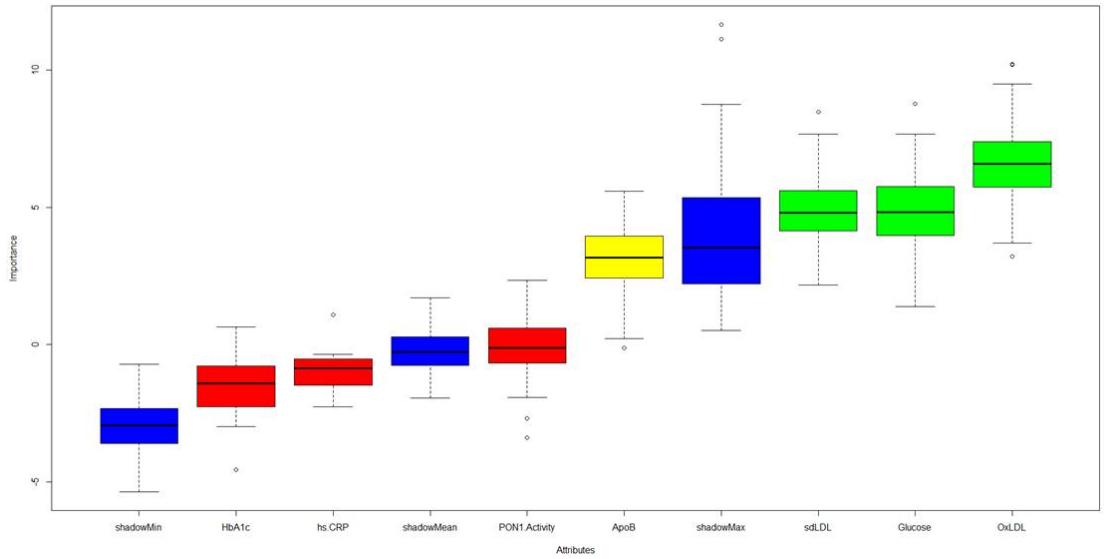

Figure 7.6: Random Forest Output for Prediction of Glycated Apo B. *HbA1c: Glycated haemoglobin; hs CRP: High-sensitivity C-reactive protein; PON1: Paraoxonase-1; Apo B: apolipoprotein B; sdLDL: small-dense low-density lipoprotein; OxLDL: Oxidized low-density lipoprotein*

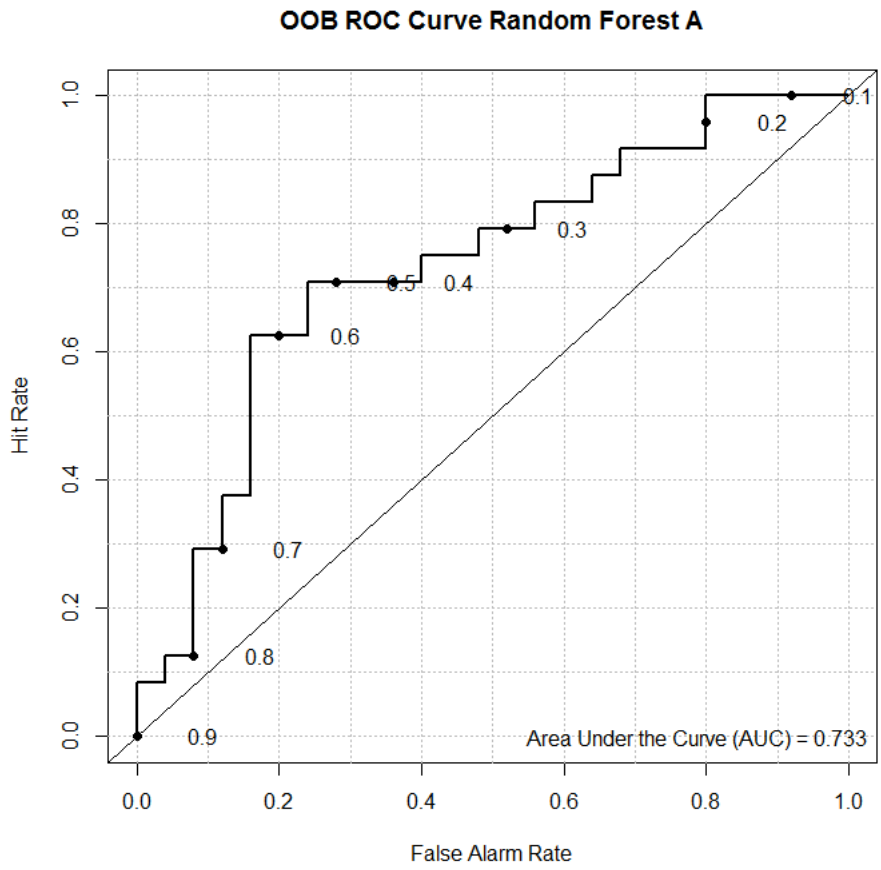

Figure 7.7: ROC Curve for Contribution of Apo B, Glucose, HbA1c, hsCRP, Oxidized LDL, PON1 and sdLDL to Glycated Apo B Concentration. *Apo B: Apolipoprotein B; HbA1c: Glycated haemoglobin; hsCRP: high-sensitivity C-reactive protein; LDL: Low-density lipoprotein; PON1: Paraoxonase-1; sdLDL: small-dense low-density lipoprotein*

8. Sphingolipids and Deoxysphingolipids in Diabetes

Abstract

Aims

Sphingolipids are a heterogeneous class of lipids derived from sphingosine that contribute to plasma membrane and lipoprotein formation. Their synthesis is typically initiated by the conjugation of L-serine and palmitoyl-CoA in a reaction catalysed by serine palmitoyltransferase. This enzyme can also use other amino acid substrates such as L-alanine and glycine, giving rise to a spectrum of atypical sphingolipids. There is mounting evidence for a role for sphingolipids in insulin resistance and diabetes. Here, we aimed to identify changes in plasma sphingoid base profiles in type 1 and type 2 diabetes, to explore their potential as biomarkers.

Methods

We compared the plasma profiles of thirteen sphingoid bases in healthy individuals (*n*=54) with those of patients with type 1 (*n*=59) and type 2 diabetes (*n*=40). Sphingoid base profiles were analyzed by Liquid Chromatography / Mass Spectrometry.

Results

Deoxysphingolipids were significantly elevated in patients with type 2 diabetes compared with controls but did not differ between patients with type 1 diabetes and healthy volunteers. Levels of sphingosine and C18-sphingadiene were significantly lower in patients with type 2 diabetes while levels of C18-phytosphingosine were elevated and C20-sphinganine levels were lower in patients with type 1 diabetes. C16-sphinganine levels were significantly lower in patients with diabetic neuropathy on a background of type 1 diabetes and C20-sphingosine levels were lower in patients with diabetic neuropathy on a background of type 2 diabetes compared both to patients with diabetes but no neuropathy and to healthy volunteers. We did not identify any association between deoxysphingolipid concentrations and macrovascular complications in type 1 diabetes as assessed by coronary artery calcification scores and carotid intima media thickness. We did not identify any association between retinopathy and sphingoid or deoxysphingoid base profiles in type 1 or type 2 diabetes, but did demonstrate lower levels of C17-sphingosine and C19-sphingosine in patients with type 1 diabetes and microalbuminuria, and higher levels of C17-sphinganine in patients with type 2 diabetes and microalbuminuria. In patients with type 2 diabetes, microalbuminuria was also associated with higher 1-deoxysphingosine concentrations.

Conclusions

We showed that deoxysphingolipids are significantly elevated in patients with type 2 diabetes, but not in patients with type 1 diabetes mellitus compared with healthy controls. They may be useful novel biomarkers to improve risk prediction and therapy monitoring in these patients.

Introduction

Sphingolipids are a structurally and functionally heterogeneous class of bioactive lipids including free sphingoid bases, ceramides, sphingomyelins, and glycosphingolipids [523]. They contribute to plasma membrane and lipoprotein formation and are involved in the development of atherosclerosis [524, 525]. Sphingolipids represent about 4% of total plasma lipids and are important signalling molecules in a number of cellular processes [526-530]. They are formed by the condensation of L-serine and palmitoyl-CoA. This first and rate-limiting step in the *de novo* synthesis of sphingolipids is catalyzed by the enzyme serine palmitoyltransferase (SPT) [524, 531-533]. The product 3-ketosphinganine is reduced to sphinganine (C18SA) and subsequently N-acylated to dihydroceramides prior to conversion to ceramides, the building blocks for the synthesis of complex sphingolipids. In the degradation pathway, ceramides are hydrolyzed to sphingosine (C18SO) [26].

The traditional carbohydrate-centred view of the pathogenesis of diabetes has widened in recent years to include different classes of lipids and inflammatory factors [534-537]. This is highly relevant to *de novo* sphingolipid synthesis, which physiologically represents a metabolic cross point, interconnecting fatty acid, amino acid and thereby indirectly also carbohydrate metabolism [524].

SPT is able to metabolize other acyl-CoAs in the range C12 to C18 without any recognised pathological effects [533, 538]. Under certain conditions however, SPT can also use the competing amino acids L-alanine and glycine as alternative substrates, forming a broad spectrum of atypical 1-deoxysphingoid bases [539, 540]. In particular, conjugation with L-alanine instead of serine forms 1-deoxysphinganine (1-deoxySA), as shown in Figure 8.1. Conjugation with glycine forms 1-deoxymethylsphinganine [541]. These two metabolites can be N-acylated to form neurotoxic 1-deoxysphingolipids which lack the C1 hydroxyl group of regular sphingoid bases and therefore cannot form complex sphingolipids such as sphingomyelin or glycosphingolipids [523, 540, 542]. 1-DeoxySA is converted to 1-deoxy(dihydro)ceramides and 1-deoxysphingosine (1-DeoxySO) but cannot be further metabolized to complex sphingolipids or efficiently degraded by the canonical pathway as the catabolic intermediate sphingosine-1-phosphate cannot be formed; thus they tend to accumulate once produced [543-545].

Gain of function missense mutations in the SPT genes *SPTLC1* and *SPTLC2* are responsible for the rare hereditary sensory and autonomic neuropathy type 1 (HSAN1) [539, 546-548]. These mutations greatly increase the irregular activity of SPT with L-alanine and glycine resulting in pathologically increased 1-deoxysphingolipid levels [539, 540, 546, 547]. HSAN1 is a length dependent axonopathy, predominantly affecting the distal extremities and characterized by progressive sensory loss giving rise to neuropathic pain and ulceration,

features more commonly associated with diabetic sensory neuropathy (DSN) [30, 539, 549-551].

Importantly, in addition to the *in vivo* effects observed in patients with HSAN1, 1-deoxysphingolipids display dose-dependent cytotoxic properties *in vitro* toward several cell lines, with altered cytoskeletal dynamics and disturbed neurite outgrowth in cultured dorsal root ganglion neurons [539, 545, 552-555]. The molecular pathways that drive 1-deoxySA-induced neurotoxicity are largely unknown but altered NMDAR activity appears to have a role [556, 557].

Fluctuations in fatty acid and thereby acyl-CoA concentrations are reflected in altered sphingoid base carbon-chain compositions [558, 559]. Altered triglyceride levels might therefore reasonably be expected to affect sphingoid base profiles, but previous studies have reported a strong positive correlation between plasma levels of triglycerides and 1-deoxysphingolipids, but not the serine-based sphingolipids [560]. This correlation is less expected, as deoxysphingolipids are defined by their alanine moiety rather than their carbon chain [26].

There is increasing evidence to support roles for sphingolipids in insulin resistance and diabetes [526, 545, 561-564]. Significantly elevated plasma levels of deoxysphingolipids have been reported in patients with metabolic syndrome and type 2 diabetes (T2DM), and levels have even been reported to be predictive for future risk of developing T2DM [538, 543, 545, 560, 565]. Atypical sphingolipids also interfere with pancreatic beta cell survival and insulin secretion, suggesting a possible role in the pathogenesis of type 1 diabetes (T1DM) as well as T2DM [545]. Considering the clinical and biochemical parallels between HSAN1 and DSN, it seems likely that deoxysphingolipids might also contribute to the development of neuropathy in T2DM [565].

Here we extend previous work by comparing the sphingoid base profiles and plasma 1-deoysphingolipid levels of individuals with T1DM, T2DM and healthy controls, examine the relationship between these parameters and diabetic neuropathy, and explore the potential of atypical sphingoid bases as biomarkers for cardiovascular disease in patients with T1DM.

Methods

After gaining written informed consent, all patients and controls were examined and fasting blood samples obtained at the Wellcome Trust Clinical Research Facility at Central Manchester University Hospitals or the Department of Diabetes & Endocrinology at the Bradford Institute for Health Research. The study was performed according to the Declaration of Helsinki and was approved by the National Research Ethics Service.

All participating patients and healthy controls were reviewed to assess basic demographic details, type and duration of diabetes, co-morbid conditions including hypertension, dyslipidaemia, known complications of diabetes, insulin and lipid-lowering medication use, and the use of tobacco and alcohol. The age range was 18–70 years. Patients with a history of coronary heart disease were excluded. Measurements were taken for height, weight and blood pressure. Healthy controls were defined by normal glucose tolerance, normal blood pressure, no use of lipid-lowering or anti-hypertensive medications, and no personal history of cardiovascular disease or diabetes. Demographic and clinical characteristics of the study participants are shown in Table 8.1.

Venous blood samples were collected after overnight fasting and serum and EDTA-plasma were isolated by centrifugation at 2000 x G for 15 minutes at 4°C within 2 hours of collection. Samples were aliqouted, anonymized and stored at 4°C until all clinical laboratory testing was complete. Remaining samples were frozen to −80°C.

Glycated haemoglobin (HbA1c) was analyzed by high-performance liquid chromatography on a VARIANT II TURBO Hemoglobin Testing System (Bio-Rad, Hercules, CA, USA). Cholesterol and triglycerides were determined by enzymatic hydrolysis and precipitation using cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) and glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) methods respectively (ABX Horiba-UK). High-density lipoprotein cholesterol (HDL-C) was measured by a direct second-generation homogeneous method (Roche Diagnostics) and low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald Formula. A Cobas Mira autoanalyzer (ABX Horiba-UK) was used for all of these assays.

For sphingoid base analysis the samples were shipped overnight on dry ice to the University of Zurich. Unless otherwise stated solvents and reagents were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) excluding methanol, which was purchased from Honeywell specialty chemicals (Seelze GmBH, Germany). Prior to analysing the sphingoid base composition, the extracted plasma sphingolipids were subjected to an acid / base hydrolysis step to release the free sphingoid bases from the conjugated N-acyl chains and headgroups. Briefly, 0.5 ml methanol including 200 pmol of the internal standards d7-

sphingosine and d7-sphinganine (d7SO, d7SA; Avanti Polar Lipids, Alabaster, AL) was added to 100 µl of plasma and extracted for 1 hour under constant agitation on a thermo-mixer at 37°C. Precipitated proteins were pelleted by centrifugation, and the supernatant transferred to a new tube. For lipid hydrolysis, 75µl of methanolic HCl (1 N HCl and 10 M H_2O in methanol) was added to the supernatant and incubated for 16 h at 65°C. This was followed by the addition of 100 µl of 10M KOH to neutralize the HCl and hydrolyze the phospholipids. To this mix, 625 µl chloroform was added, followed by 100 µl 2N ammonium hydroxide and 0.5 ml alkaline water to complete the phase separation. The mix was then vortexed and centrifuged at 16,000 x G for 5 minutes. After centrifugation, the upper phase was discarded and the lower organic phase washed 2-3 times with alkaline water (pH 10.3). Finally, the organic phase was dried under N_2 and kept at -20°C until analysis.

The sphingoid base profile was analyzed by Liquid Chromatography / Mass Spectrometry (LC-MS) as described previously [539, 540, 545, 560]. The sphingoid bases were separated on a C18 column (Uptispere 120 A, 5 µm, 125 x 2 mm; Interchim, Montlucon, France) and analyzed on a TSQ Quantum Ultra mass spectrometer (Thermo, Reinach, BL, Switzerland). Each sample was measured as a singleton [26]. Intra- and inter-assay coefficient of variation of the method was between 5% and 20%. Analyzed sphingoid bases included C16-Sphingosine (C16SO), C16-Sphinganine (C16SA), C17-Sphingosine (C17SO), C17-Sphinganine (C17SA), C18SO, C18SA, C18-Phytosphingosine (C18PhytoSO), C18-Sphingadiene (C18SAdiene), C19-Sphingosine (C19SO), C20-Sphingosine (C20SO), C20-Sphinganine (C20SA), 1-deoxySO and 1-deoxySA.

Patients with T1DM or T2DM were evaluated for the presence of DSN using a modified neuropathy disability score (NDS) with assessment of vibration perception using a 128-Hz tuning fork, ankle reflex testing, temperature differentiation and pinprick testing. A score of 0 was given for a normal response and 1 for an abnormal response for each individual test component (except ankle reflex testing, where a score of 1 indicates presence with reinforcement, and 2 complete absence). Thus the maximum score is 10, with an NDS of ≥ 3 indicative of peripheral neuropathy [30, 31]. All parameters were assessed by the same two examiners. The presence of large fibre neuropathy was evaluated by vibration perception threshold testing (VPT) using a Neurothesiometer (Horwell; Scientific Laboratory Supplies, Nottingham, UK) and nerve conduction studies for selected patients (Dantec Dynamics, Bristol, UK). The presence of small fibre neuropathy was evaluated by quantitative sensory testing using a TSA-II NeuroSensory Analyzer (Medoc, Ramat-Yishai, Israel) and corneal confocal microscopy (CCM) using a Heidelberg Retinal Tomograph III Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany). Neuropathy was diagnosed through CCM according to known age-adjusted normative values for corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD) and corneal nerve fibre length (CNFL) [32]. These assessments were performed by a single examiner.

Retinopathy was graded according to results from the most recent attendance under the NHS diabetic eye screening programme. The presence of microalbuminuria was assessed by measurement of albumin-to-creatinine ratio in a random spot urine collection [437].

40 patients with T1DM underwent Coronary Artery Calcification (CAC) Score determination by electron-beam CT using an automated program based on the Agatson method as a screening tool to identify subclinical atherosclerosis [27]. A CAC Score >10 was considered significant [28]. The carotid arteries were imaged in the remaining patients with T1DM with a Siemens Sequoia ultrasonography system (Siemens Medical Solutions, Mountain View, CA) with an 8- to 15-MHz linear array transducer. Examination included measurement of common and internal carotid artery flow velocities and Carotid Intima-Media thickness (CIMT) at each of 3 scan planes. Here a mean CIMT of 0.06 cm was considered significant [29].

The clinical and lipid data were merged and clinical baseline characteristics and sphingoid base levels compared between groups (controls, T1DM, T2DM) by univariate and multivariate linear regression models to control for baseline differences between groups. Normal distribution was tested for all data with the Kolmogorov-Smirnov, D'Agostino and Pearson omnibus and Shapiro-Wilk normality tests. A Bonferroni correction to account for multiple testing in multivariate model building was applied, with a significance level of 0.05, which corresponds to a p-value of 0.004. All analyses were performed with SPSS statistical software, version 19.0 (IBM, Zurich, Switzerland). Patients with T1DM and T2DM were subdivided according to NDS, CCM or clinical assessment of neuropathy, according to CAC scores and CIMT measurements, and according to the presence of microalbuminuria or retinopathy grading. Significance was assessed with Student unpaired, two-tailed t tests or one-way ANOVA. Non-linear regression analysis was performed comparing the 1-deoxysphingolipid levels in plasma with NDS, CCM parameters (Corneal Nerve Fibre Density [CNFD], Corneal Nerve Branch Density [CNBD], and Corneal Nerve Fibre Length [CNFL], CAC score, and carotid intima media thickness (CIMT).

Results

For this study, we analyzed plasma samples from a total of 153 individuals with T1DM (N = 59), T2DM (N = 40) and healthy volunteers (HV) without diabetes or evidence of metabolic syndrome (N = 54). Unadjusted demographic and clinical characteristics are shown in Table 1.

There were significant differences between the three groups. The T2DM group was on average older (57 +/- 10.8 years) and had higher triglyceride (1.88 +/- 1.30 mmol/l) and

lower HDL-C levels (1.25 +/- 0.44 mmol/l) but lower total cholesterol (4.18 +/- 1.12 mmol/l) associated with more prevalent statin use. Previous studies have shown plasma 1-deoxysphingolipids (1-deoxySA and 1-deoxySO) to be significantly increased in patients with dyslipidaemia [560], so univariate and multivariate regression models were used to control for these baseline differences in the lipid profile. Here the healthy volunteers had an apparently more adverse lipid profile than patients with T1DM. No significant difference in HbA1c was seen between patients with T1DM (64 +/- 12 mmol/mol) and T2DM (63 +/- 20 mmol/mol). Twenty-seven of the 59 patients with T1DM were using an insulin pump. The median (and mean) duration of diabetes was 18 years (4-44 years) in patients with T1DM and five patients had been prescribed statin therapy. The median duration of diabetes in patients with T2DM was 13 years (1-39 years). Nineteen of the 40 patients with T2DM were on insulin therapy and thirty-two had been prescribed lipid-lowering medications.

For all measured sphingoid bases (Table 8.2), the level of the unsaturated form (sphingosine [SO]) was higher than that of the saturated form (sphinganine [SA]). The most abundant sphingoid base in plasma was C18SO, followed by C18SAdiene, C16SO and C17SO. The absolute amounts of 1-deoxysphingolipids were minor compared to the total distribution of plasma sphingoid bases, but differed significantly between the examined groups. The sphingoid bases formed in the canonical SPT reaction are C18SA, C18SO, and C18SAdiene [524]. In keeping with previous reports, C18SO and C18SAdiene were significantly lower in T2DM and C18SA was by trend lower in T1DM compared to controls [543, 560]. Here C18PhytoSO was significantly higher in patients with T1DM. For the sphingoid bases with atypical chain lengths, C16SA was previously shown to be significantly lower in T1DM compared to T2DM or controls [524], but was non-significantly lower here. C20SA was lower in T1DM, although this change missed the significance limit after Bonferroni correction. Concentrations of other sphingoid bases were not significantly different between the three groups. C17SO levels were also observed to be significantly higher in patients with T1DM compared to patients with T2DM.

In contrast, 1-deoxysphingolipid levels were clearly different between the 3 groups (Figure 8.2). In agreement with previous reports, 1-deoxysphingolipids (1-deoxySO and 1-deoxySA) were significantly elevated in T2DM [524, 560]. After controlling for age, Triglycerides and LDL-C, 1-deoxysphingolipid levels remained significantly elevated in T2DM compared both to controls and to patients with T1DM. No difference in levels was seen between T1DM and controls.

The plasma sphingoid base profiles were also analysed in patients with T1DM and T2DM according to qualitative or quantitative evidence of the presence or absence of DSN (Tables 8.3 & 8.4). Here there did not appear to be any significant correlation between the presence of neuropathy and plasma 1-deoxysphingolipid levels. Interestingly, given that

there is no difference in 1-deoxysphingolipid levels between healthy volunteers and patients with T1DM, patients with T1DM and DSN defined by a NDS of ≥ 3 did exhibit higher 1-deoxysphingolipid levels than patients with T1DM and NDS < 3, although this difference failed to reach statistical significance. A previous comparison of neuropathy subgroups and unaffected individuals also failed to show a significant correlation of 1-deoxysphingolipid plasma levels to disease severity [566].

C20SO plasma levels have previously been shown to be significantly lower in patients with small fibre neuropathy compared to healthy controls [566]. Here we showed C20SO levels to be lower in patients with diabetic neuropathy on a background of T2DM compared both to patients with diabetes but no neuropathy and to healthy volunteers. In particular, lower C20SO levels were associated with reductions in all parameters assessed by CCM with the decrease in CNBD approaching significance.

We did not identify any association between retinopathy and sphingoid or deoxysphingoid base profiles in T1DM or T2DM, but did demonstrate lower levels of C17SO (2.60 +/- 0.71 µmol/l *vs*. 3.73 +/- 1.23 µmol/l) and C19SO (0.67 +/- 0.32 µmol/l *vs*. 1.10 +/- 0.44 µmol/l) in patients with T1DM microalbuminuria and higher levels of C17SA (0.08 +/- 0.04 µmol/l *vs*. 0.05 +/- 0.01 µmol/l) in patients with T2DM microalbuminuria. In patients with T2DM, microalbuminuria was also associated with higher 1-deoxySO concentrations (0.16 +/- 0.06 µmol/l *vs*. 0.11 +/- 0.04 µmol/l).

Finally, we also looked to establish an association between the plasma sphingoid base profile and cardiovascular risk in patients with T1DM measured by coronary artery calcification (CAC) or carotid intima media thickness (Tables 8.5 & 8.6). 33 of 40 patients had a CAC score of zero. A CAC score of >10 has been shown to predict cardiovascular events in people with and without diabetes, but we did not demonstrate any association between sphingolipid or 1-deoxysphingolipid levels in patients with T1DM and a CAC score of >10 compared to patients with T1DM and minimal calcification [28, 567].

A CIMT < 0.06 cm has been shown to be associated with few incident cardiovascular events [29]. Here CIMT measurements > 0.06 cm were associated with lower levels of C18SA, C20SO and C20SA.

In accordance with earlier observations, we found a significant positive correlation of 1-deoxysphingolipids with triglycerides (Figure 8.3), glucose and HbA1c whereas the serine-derived sphingoid bases correlated positively with LDL-C and total cholesterol [543, 560]. These relationships were less apparent for patients with T1DM compared to healthy volunteers or those with T2DM.

Discussion

1-deoxysphingolipids are atypical and neurotoxic products formed by SPT due to the alternate use of L-alanine over its canonical substrate L-serine [26, 524]. Pathologically elevated 1-deoxysphingolipid formation is found in the rare inherited neuropathy HSAN1, a disease associated with gain-of-function mutations in SPT which alter enzyme substrate specificity [524, 539, 546, 568, 569]. 1-deoxysphingolipids are also present at low levels in the plasma of healthy individuals but at significantly elevated levels in individuals with metabolic syndrome and T2DM, raising the possibility of a role in the development of these pathologies [543, 545, 560]. However, here increased 1-deoxysphingolipid levels are associated with derailed carbohydrate and fatty acid metabolism rather than being the result of mutations in SPT [26]. The reason why these lipids are formed preferentially under certain conditions is not yet understood, although a possible mechanism might be increased availability of intracellular alanine in hyperglycaemic states [524]. Sphingolipid metabolism can thus be considered a metabolic cross point, connecting amino acid (serine and alanine) and fatty acid (acyl-CoA) metabolism.

The mechanism underlying the augmented production of 1-deoxysphingolipids in T2DM remains unclear. In this work we compared 1-deoxysphingolipid levels between individuals with T1DM, T2DM and healthy controls. In agreement with previous reports, we confirmed elevated 1-deoxysphingolipids in T2DM [524, 543]. However, 1-deoxysphingolipid levels were not increased in T1DM. As glucose and HbA1c levels were not significantly different between the T1DM and T2DM groups it would appear that hyperglycaemia *per se* may not directly determine 1-deoxysphingolipid formation. These findings stand in contrast to the observation that 1-deoxysphingolipids are elevated in STZ (streptozotocin) rats, a T1DM animal model [560].

Alternatively, altered 1-deoxysphingolipid levels may be driven by triglyceride levels. T2DM but not T1DM is typically associated with elevated triglyceride levels. A functional link between plasma triglyceride levels and 1-deoxysphingolipid formation might explain elevated 1-deoxysphingolipids in the STZ rat model which typically has a more atherogenic lipid profile (including elevated plasma triglyceride levels) than human T1DM patients [524]. The correlation between 1-deoxysphingolipid and triglyceride levels shown here has been demonstrated previously [543, 560], but the lack of correlation in patients with T1DM merits further consideration. Similarly, serine-based sphingoid bases showed a closer correlation to total and LDL cholesterol levels [524], but this relationship was again less clear in patients with T1DM.

It is interesting that Fenofibrate is able to specifically lower 1-deoxysphingolipids in patients with dyslipidaemia while Niacin has no effect, as both treatments lower

triglyceride levels, LDL-C, and total cholesterol. However, this is also unlikely to be a direct effect as it is not fatty acid levels but rather the conjugation of L-alanine, instead of L-serine, which defines the formation of 1-deoxysphingolipids, and the mechanism influencing the use of alanine by SPT remains unknown [26].

Fluctuations in fatty acid and thereby acyl-CoA concentrations are reflected in altered sphingoid base carbon-chain compositions [558, 559]. Here we observed significantly lower levels of C18SO and C18SAdiene in patients with T2DM compared to healthy volunteers, in keeping with previous reports [543, 560]. We also report significantly higher levels of C18PhytoSO in patients with T1DM compared to healthy volunteers. Of the sphingoid bases with atypical chain lengths, we observed lower levels of C20SA in patients with T1DM compared to healthy volunteers and significantly higher C17SO levels in patients with T1DM compared to patients with T2DM but concentrations of other sphingoid bases were not significantly different between the three groups. Further work is required to establish whether these altered sphingoid base profiles are relevant to the development and progression of both T2DM and T1DM.

As the clinical pictures of DSN and HSAN1 distinctly resemble each other, it is reasonable to consider that elevated 1-deoxysphingolipid levels might be a common factor in the aetiology of both conditions. Here there did not appear to be any significant correlation between the presence of neuropathy and plasma 1-deoxysphingolipid levels in patients with T1DM or T2DM. Interestingly, given that there is no difference in 1-deoxysphingolipid levels between healthy volunteers and patients with T1DM, patients with T1DM and DSN defined by a NDS of ≥ 3 did exhibit higher 1-deoxysphingolipid levels than patients with T1DM and NDS < 3, although this difference failed to reach statistical significance. A previous comparison of neuropathy subgroups and unaffected individuals also failed to show a significant correlation of 1-deoxysphingolipid plasma levels to disease severity [566].

Targeting 1-deoxysphingolipid synthesis as a therapeutic strategy for T2DM and its complications still warrants further investigation. It is important to note that the exact point of onset is difficult to determine for diabetic neuropathy. In the UK Prospective Diabetes Study 5-7% of patients already had neuropathy at the time of diagnosis with T2DM [570]. Even early neuronal damage might be linked to the neurotoxicity of deoxysphingoid bases, and our failure here to show any significant correlation between 1-deoxysphingolipid levels and the degree of neuropathy assessed by NDS or CCM may reflect the prevalence of subclinical neuropathy in our cohort.

Both concentration and time and dependent neurotoxic effects of 1-deoxySA on primary cortical neurons have been reported, and it seems likely that it is overall 1-deoxysphingolipid exposure which alters neuronal cytoskeletal architecture rather than

isolated high levels. Increased 1-deoxysphingolipid plasma levels therefore do not appear to correlate with the clinical course of DSN, which might be influenced by other confounding factors. Serum triglycerides do appear to correlate with the progression of DSN, and in *post hoc* analyses from randomized clinical trials and longitudinal cohorts, Fenofibrate has been associated with improvements in DSN and other microvascular complications [571-573]. These effects may of course be related to the effect of Fenofibrate on 1-deoxysphingolipids, and the mechanism of any improvement in DSN requires further investigation [26]. 1-deoxysphingolipid-induced neuronal death is also likely to be mediated by 1-deoxyceramide species.

Plasma C20SO levels have previously been reported to be significantly lower in patients with a small fibre neuropathy [566]. Mechanisms underlying the development of small fibre neuropathy are complex and often unknown, and it is considered to represent the earliest change in the spectrum of diabetic neuropathy [574]. Here we showed C20SO levels to be lower in patients with DSN on a background of T2DM compared both to patients with diabetes but no clinical evidence of neuropathy and to healthy volunteers. CCM allows quantitative assessment of small fibre neuropathy, and lower C20SO levels were associated with reductions in all parameters assessed by CCM with the change in CNBD approaching significance.

Future research around 1-deoxysphingolipids should explore both their potential use as biomarkers and their role in pathophysiological processes, which might ultimately lead to novel treatment options. Oral supplementation with L-serine suppresses the formation of 1-deoxysphingolipids and prevents neuropathy in HSAN1, with protective effects confirmed in animal models [565, 575, 576]. Moreover, 1-deoxySA activates the NMDA receptor and NMDA receptor blockade prevents 1-deoxySA-induced neuronal death [541]. In this light, it is very interesting to note that the use of NMDA receptor antagonists was also recently suggested in the treatment of diabetes [577]. Glutamate receptor inhibitors may also prevent 1-deoxysphingolipid-induced neurodegeneration and offer a novel therapeutic approach in HSAN1 and DSN [541].

Here we also investigated the association of plasma sphingoid bases with atherosclerosis and their ability to predict macrovascular complications in diabetes by correlation with CAC scores and CIMT in patients with T1DM. Low levels of C18SAdiene and increased levels of C20SO have recently been shown to be independent and predictive biomarkers for future cardiovascular events but we did not demonstrate any association between any sphingoid base levels and CAC score in patients with T1DM [578]. Furthermore, in this study, increased CIMT was found to be associated with lower levels of C18SA, C20SO and C20SA. Elucidating the underlying mechanisms which link alterations in sphingolipid metabolism with increased cardiovascular risk is of scientific and clinical importance, but may not

explain the increased cardiovascular risk associated with T1DM.

Sphingolipid metabolism and the contribution of 1-deoxysphingolipids to T1DM and its complications would appear to be more complex than the interactions currently being explored in T2DM, a view supported by the less well defined relationships between sphingoid bases, 1-deoxysphingolipids, glycaemia and dyslipidaemia in T1DM.

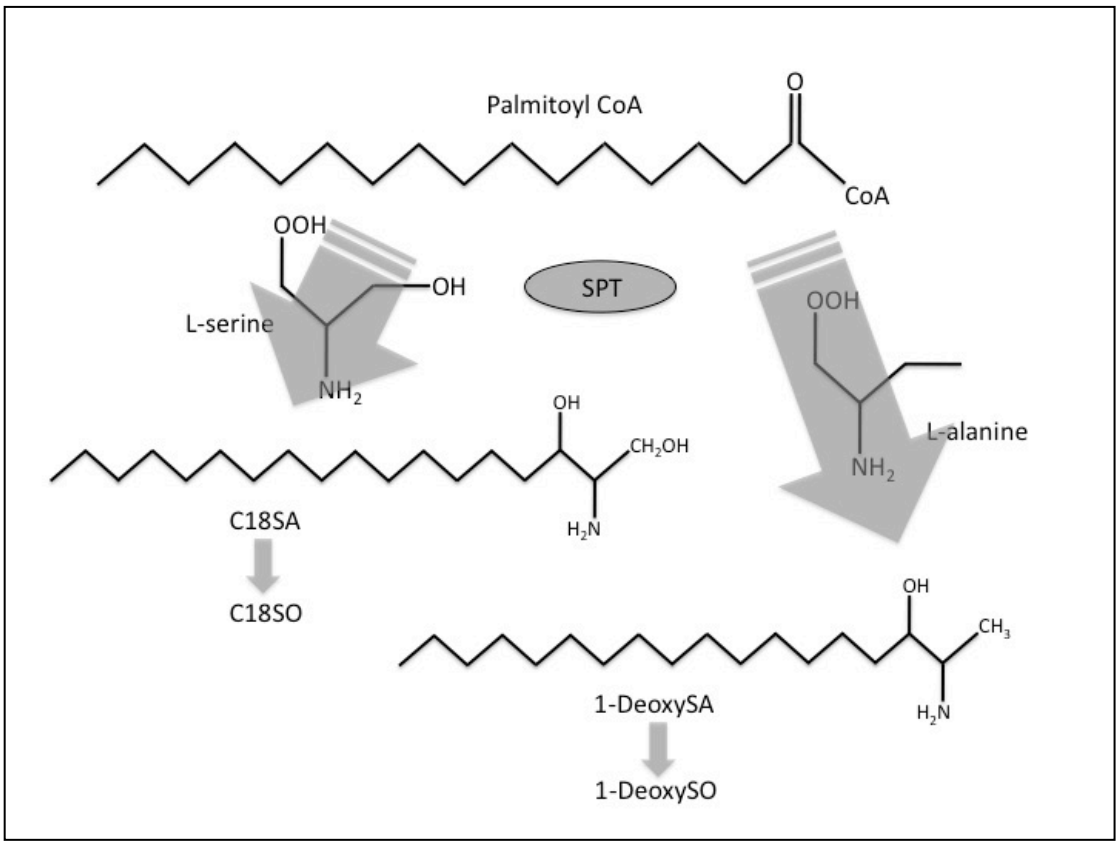

Figure 8.1: *de novo* sphingolipid and 1-deoxysphingolipid synthesis. *SPT: Serine palmitoyltransferase; C18SA: Sphinganine; C18SO: Sphingosine; 1-DeoxySA: 1-Deoxysphinganine; 1-DeoxySO: 1-Deoxysphingosine*

	HV	T1DM	T2DM
Age (years)	41.8 +/- 10.2	39.3 +/- 11.9	57.3 +/- 10.8
Gender (% Male)	33.33	44.07	72.5
BMI (kg/m^2)	25.10 +/- 3.79	25.97 +/- 4.24	31.38 +/- 5.36
Systolic BP (mmHg)	117.8 +/- 15.9	122.9 +/- 15.5	133.4 +/- 17.1
Diastolic BP (mmHg)	73.5 +/- 10.1	76.2 +/- 10.3	72.6 +/- 10.4
Fasting Glucose (mmol/l)	4.53 +/- 0.66	9.19 +/- 4.41	9.17 +/- 5.26
HbA1c (mmol/mol)	-	64.0 +/- 12.0	62.7 +/- 20.4
Total Cholesterol (mmol/l)	5.41 +/- 1.05	4.84 +/- 0.73	4.18 +/- 1.12
Triglycerides (mmol/l)	1.41 +/- 0.83	0.97 +/- 0.40	1.88 +/- 1.30
HDL-C (mmol/l)	1.40 +/- 0.36	1.70 +/- 0.44	1.25 +/- 0.44
LDL-C (mmol/l)	3.36 +/- 0.80	2.72 +/- 0.60	2.06 +/- 0.86

Table 8.1: Demographic and Clinical Characteristics and Results. *HV: Healthy Volunteers; T1DM: Type 1 Diabetes; T2DM: Type 2 Diabetes; BMI: Body Mass Index; BP: Blood Pressure; HbA1c: Glycated Haemoglobin; HDL-C: High-Density Lipoprotein Cholesterol; LDL-C: Low-Density Lipoprotein Cholesterol.* Values shown as mean +/- standard deviation

	HV (µmol/l)	T1DM (µmol/l)	Significance *(HV vs. T1DM)*	T2DM (µmol/l)	Significance *(HV vs. T2DM)*	Significance *(T1DM vs. T2DM)*
C16SO	7.19 +/- 3.01	8.08 +/- 3.97	*NS*	6.83 +/- 2.59	*NS*	*NS*
C16SA	0.24 +/- 0.13	0.20 +/- 0.09	*NS*	0.22 +/- 0.11	*NS*	*NS*
C17SO	3.52 +/- 1.34	3.62 +/- 1.24	*NS*	3.06 +/- 1.01	*NS*	*P = 0.020*
C17SA	0.06 +/- 0.02	0.06 +/- 0.03	*NS*	0.06 +/- 0.02	*NS*	*NS*
C18SO	42.59 +/- 14.7	42.78 +/- 10.5	*NS*	36.84 +/- 9.43	*P = 0.033*	*P = 0.005*
C18PhytoSO	0.09 +/- 0.03	0.08 +/- 0.03	*P = 0.042*	0.09 +/- 0.03	*NS*	*NS*
C18SA	1.62 +/- 0.53	1.51 +/- 0.43	*NS*	1.57 +/- 0.72	*NS*	*NS*
C18SAdiene	16.07 +/- 4.35	16.44 +/- 5.13	*NS*	13.18 +/- 3.40	*P < 0.001*	*P < 0.001*
C19SO	1.12 +/- 0.53	1.06 +/- 0.45	*NS*	0.92 +/- 0.39	*NS*	*NS*
C20SO	0.11 +/- 0.04	0.11 +/- 0.03	*NS*	0.11 +/- 0.05	*NS*	*NS*
C20SA	0.02 +/- 0.01	0.02 +/- 0.01	*P = 0.047*	0.02 +/- 0.01	*NS*	*NS*
1-DeoxySO	0.09 +/- 0.04	0.09 +/- 0.04	*NS*	0.13 +/- 0.04	*P < 0.001*	*P < 0.001*
1-DeoxySA	0.03 +/- 0.02	0.03 +/- 0.01	*NS*	0.05 +/- 0.03	*P < 0.001*	*P < 0.001*

Table 8.2: Plasma sphingoid and deoxysphingoid base concentrations in patients with and without Diabetes. *HV: Healthy Volunteers; T1DM: Type 1 Diabetes; T2DM: Type 2 Diabetes; SO: Sphingosine; SA: Sphinganine; SAdiene: Sphingadiene; NS: non-significant*. Values shown as mean +/- standard deviation

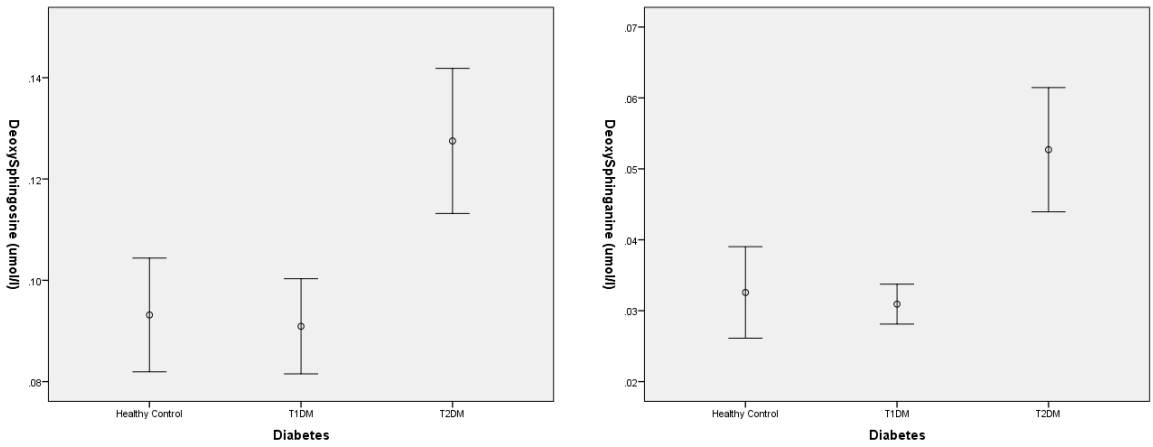

Figure 8.2: 1-deoxysphingolipid levels in healthy volunteers and patients with T1DM or T2DM. Error bars represent 95% Confidence Interval

	NDS	CNFD (/mm^2)	CNBD (/mm^2)	CNFL (mm/mm^2)	Retinopathy	Microalbuminuria (mg/mmol)
C16SO	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
C16SA	*NS*	*P = 0.016*	*NS*	*NS*	*NS*	*NS*
C17SO	*NS*	*NS*	*NS*	*NS*	*NS*	*P = 0.049*
C17SA	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
C18SO	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
C18PhytoSO	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
C18SA	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
C18SAdiene	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
C19SO	*NS*	*NS*	*NS*	*NS*	*NS*	*P = 0.038*
C20SO	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
C20SA	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
1-DeoxySO	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
1-DeoxySA	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

Table 8.3: Differences in plasma sphingoid and deoxysphingoid bases in patients with T1DM with and without neuropathy assessed by NDS / CCM, with and without retinopathy, and with and without microalbuminuria. *NDS: Neuropathy Disability Score; CCM: Corneal Confocal Microscopy; CNFD: Corneal Nerve Fibre Density; CNBD: Corneal Nerve Branch Density; CNFL: Corneal Nerve Fibre Length*

	Clinical Evidence of Neuropathy	Retinopathy	Microalbuminuria
C16SO	*NS*	*NS*	*NS*
C16SA	*NS*	*NS*	*NS*
C17SO	*NS*	*NS*	*NS*
C17SA	*NS*	*NS*	*P = 0.018*
C18SO	*NS*	*NS*	*NS*
C18PhytoSO	*NS*	*NS*	*NS*
C18SA	*NS*	*NS*	*NS*
C18SAdiene	*NS*	*NS*	*NS*
C19SO	*NS*	*NS*	*NS*
C20SO	*P = 0.044*	*NS*	*NS*
C20SA	*NS*	*NS*	*NS*
1-DeoxySO	*NS*	*NS*	*P = 0.037*
1-DeoxySA	*NS*	*NS*	*NS*

Table 8.4: Differences in plasma sphingoid and deoxysphingoid bases in patients with T2DM with and without clinical evidence of neuropathy, retinopathy or microalbuminuria

	CAC Score <10	CAC Score >10	Significance
C16SO (µmol/l)	7.58 +/- 2.51	6.60 +/- 1.83	*NS*
C16SA (µmol/l)	0.19 +/- 0.08	0.20 +/- 0.06	*NS*
C17SO (µmol/l)	3.49 +/- 0.95	3.74 +/- 1.05	*NS*
C17SA (µmol/l)	0.06 +/- 0.03	0.05 +/- 0.01	*NS*
C18SO (µmol/l)	43.73 +/- 11.35	38.25 +/- 8.00	*NS*
C18PhytoSO (µmol/l)	0.10 +/- 0.04	0.08 +/- 0.03	*NS*
C18SA (µmol/l)	1.49 +/- 0.48	1.50 +/- 0.39	*NS*
C18SAdiene (µmol/l)	16.16 +/- 4.00	17.21 +/- 2.00	*NS*
C19SO (µmol/l)	1.08 +/- 0.40	1.12 +/- 0.51	*NS*
C20SO (µmol/l)	0.11 +/- 0.04	0.11 +/- 0.04	*NS*
C20SA (µmol/l)	0.02 +/- 0.01	0.02 +/- 0.01	*NS*
1-DeoxySO (µmol/l)	0.09 +/- 0.04	0.07 +/- 0.02	*NS*
1-DeoxySA (µmol/l)	0.03 +/- 0.01	0.02 +/- 0.01	*NS*

Table 8.5: Plasma sphingoid and deoxysphingoid bases in patients with T1DM according to CAC Score. *CAC: Coronary Artery Calcification.* Values shown as mean +/- standard deviation

	CIMT < 0.06 cm	CIMT > 0.06 cm	Significance
C16SO (µmol/l)	9.19 +/- 2.13	9.82 +/- 7.57	*NS*
C16SA (µmol/l)	0.28 +/- 0.12	0.19 +/- 0.09	*NS*
C17SO (µmol/l)	4.34 +/- 1.62	3.58 +/- 1.52	*NS*
C17SA (µmol/l)	0.08 +/- 0.02	0.06 +/- 0.02	*NS*
C18SO (µmol/l)	46.44 +/- 8.99	40.76 +/- 10.42	*NS*
C18PhytoSO (µmol/l)	0.11 +/- 0.04	0.10 +/- 0.03	*NS*
C18SA (µmol/l)	1.75 +/- 0.31	1.36 +/- 0.31	*P = 0.039*
C18SAdiene (µmol/l)	16.94 +/- 2.46	14.75 +/- 4.28	*NS*
C19SO (µmol/l)	1.33 +/- 0.48	1.02 +/- 0.58	*NS*
C20SO (µmol/l)	0.13 +/- 0.02	0.10 +/- 0.02	*P = 0.016*
C20SA (µmol/l)	0.02 +/- 0.00	0.02 +/- 0.00	*P = 0.003*
1-DeoxySO (µmol/l)	0.11 +/- 0.02	0.10 +/- 0.02	*NS*
1-DeoxySA (µmol/l)	0.04 +/- 0.01	0.03 +/- 0.01	*NS*

Table 8.6: Plasma sphingoid bases in patients with T1DM according to CIMT (Mean +/- sd). *CIMT: Carotid Intima Media Thickness. Values shown as mean +/- standard deviation*

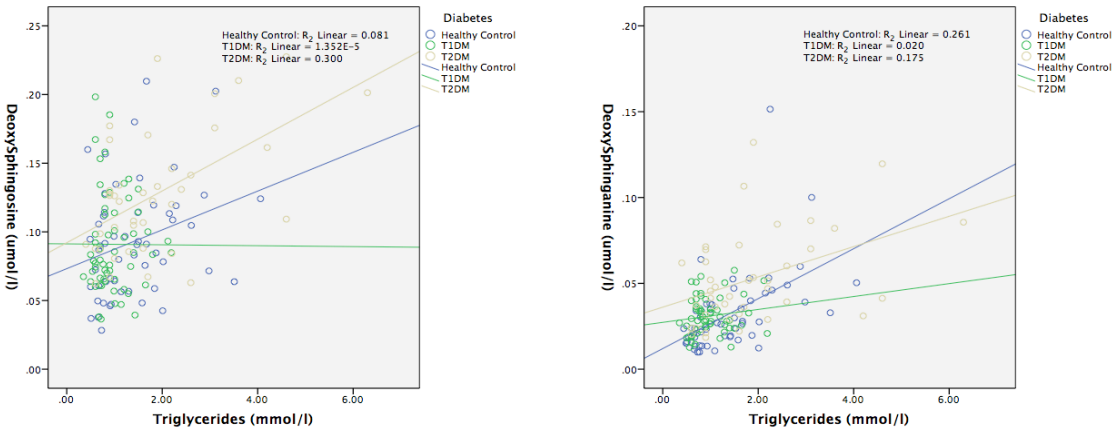

Figure 8.3: Correlation between 1-deoxysphingolipid and triglyceride levels

9. Ceramides in Diabetes

Abstract

Aims

Ceramides are principal in the sphingolipid biosynthesis and metabolism pathway. Regulation of ceramide synthesis and hydrolysis depends on a number of factors. Both sphingolipids and ceramides have wide-ranging physiological and pathological effects, including contributions to the development of atherosclerosis and insulin resistance. This study aimed to examine ceramide species in type 1 and type 2 diabetes, and their associations with high-density and low-density lipoprotein cholesterol concentrations.

Methods

Plasma ceramide species from people with type 1 diabetes (n=59), type 2 diabetes (n=40) and healthy controls (n=54) were analysed by liquid chromatography / mass spectrometry. Results were compared between groups and analysed alongside variations in glycaemia and the lipid profile between the cohorts.

Results

People with type 2 diabetes had lower plasma concentrations of all ceramide species, associated with significantly lower low-density lipoprotein cholesterol concentrations. Patients with type 1 diabetes and evidence of subclinical atherosclerosis on carotid Doppler studies had a significantly higher Cer(d18:1/16:0)/Cer(d18:1/24:0) ratio than healthy volunteers, despite a less adverse lipid profile.

Conclusions

Plasma ceramide levels can be effectively reduced with LDL-directed therapy, but a distribution of ceramide species associated with cardiovascular death persists. This study is the first report of a possible role for plasma ceramides in predicting cardiovascular outcomes in patients with type 1 diabetes.

Introduction

Sphingolipids are a structurally and functionally heterogeneous class of bioactive lipids including free sphingoid bases, ceramides, sphingomyelins, and glycosphingolipids [523]. Ceramides are principal in the sphingolipid biosynthesis and metabolism pathway [579]. Synthesis of ceramides occurs either *de novo* or via the breakdown of sphingomyelin or glycosphingolipids and sulfatites [580]. In the *de novo* pathway, they are formed by the condensation of L-serine and palmitoyl-CoA. This first and rate-limiting step is catalysed by the enzyme serine palmitoyltransferase (SPT) [524, 531-533]. The product 3-ketosphinganine is reduced to sphinganine (C18SA) and subsequently N-acylated to dihydroceramides prior to conversion to ceramides, the building blocks for the synthesis of complex sphingolipids. In the degradation pathway, ceramides are hydrolysed to sphingosine (C18SO) [26]. Regulation of ceramide synthesis and hydrolysis depends on a number of factors. Increased availability of palmitate and inflammation favour synthesis whilst adiponectin and fibroblast growth factor 21 promote ceramide degradation [581].

Sphingolipids and ceramides have wide-ranging physiological and pathological effects (Figure 9.1). They contribute to plasma membrane and lipoprotein formation and are involved in the development of atherosclerosis [524, 525]. Ceramides have been shown to induce apoptosis in the retina [582], renal tubule [583] and pancreas as well as contribute to insulin resistance in skeletal muscle and adipose tissue [581]. Ceramides have also been implicated in the development of cardiac muscle dysfunction [581]. Interestingly, a recent study by Laaksonen *et al*. showed that certain subgroups of plasma ceramides are independent predictors of cardiovascular mortality, even when considering low-density lipoprotein (LDL) cholesterol levels [584]. Long-chain ceramides (d18:1/16:0 and d18:1/18:0) were associated with higher incidence of cardiovascular death whilst very-long-chain ceramides (e.g. d18:1/24:0) were thought to have a protective effect [585].

A key area of interest is the relationship between sphingolipids (including ceramides), insulin resistance and diabetes. Insulin resistance is promoted in a ceramide-rich environment whilst inhibition of ceramide production augments insulin sensitivity [586]. Additionally, ceramides induce pancreatic beta-cell apoptosis [587]. Therefore, ceramides potentially have a key role in the development of both type 1 (T1DM) and type 2 diabetes mellitus (T2DM). Importantly, there is also evidence to suggest that ceramides may have a role in microvascular complications of diabetes as there are relationships with renal disease, retinopathy, and neuropathy [588].

The aim of this study was to quantify ceramide species and their distribution in both T1DM and T2DM. A secondary objective was to ascertain if there was any association between high-density lipoprotein (HDL) cholesterol or LDL cholesterol and different ceramide

species, and whether the distribution of ceramide species in diabetes was similar to that recently associated with adverse cardiovascular outcomes [584].

Methods

After gaining written informed consent, all patients and controls were examined and fasting blood samples obtained at the Wellcome Trust Clinical Research Facility at Central Manchester University Hospitals or the Department of Diabetes & Endocrinology at the Bradford Institute for Health Research. The study was performed according to the Declaration of Helsinki and was approved by the National Research Ethics Service.

All participating patients and healthy controls were reviewed to assess basic demographic details, type and duration of diabetes, co-morbid conditions including hypertension, dyslipidaemia, known complications of diabetes, insulin and lipid-lowering medication use, and the use of tobacco and alcohol. The age range was 18–70 years. Patients with a history of coronary heart disease were excluded. Measurements were taken for height, weight and blood pressure. Healthy controls were defined by normal glucose tolerance, normal blood pressure, no use of lipid-lowering or anti-hypertensive medications, and no personal history of cardiovascular disease or diabetes. Demographic and clinical characteristics of the study participants are shown in Table 9.1.

Venous blood samples were collected after overnight fasting and serum and EDTA-plasma were isolated by centrifugation at 2000 x G for 15 minutes at 4°C within 2 hours of collection. Samples were aliqouted, anonymized and stored at 4°C until all clinical laboratory testing was complete. Remaining samples were frozen to −80°C.

Glycated haemoglobin (HbA1c) was analyzed by high-performance liquid chromatography on a VARIANT II TURBO Hemoglobin Testing System (Bio-Rad, Hercules, CA, USA). Cholesterol and triglycerides were determined by enzymatic hydrolysis and precipitation using cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) and glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) methods respectively (ABX Horiba-UK). High-density lipoprotein cholesterol (HDL-C) was measured by a direct second-generation homogeneous method (Roche Diagnostics) and low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald Formula. A Cobas Mira autoanalyzer (ABX Horiba-UK) was used for all of these assays.

For quantification of plasma ceramides samples were shipped overnight on dry ice to the University of Zurich. Unless otherwise stated solvents and reagents were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) excluding methanol, which was purchased from Honeywell specialty chemicals (Seelze GmBH, Germany). Prior to analysing

the ceramide composition, the extracted plasma ceramides were subjected to base hydrolysis. Briefly, 1 ml methanol with chloroform (2:1) including 0.2 μl/ml of the internal standards d7-sphingosine and d7-sphinganine (d7SA, d7SO; Avanti Polar Lipids, Alabaster, AL) was added to 100 µl of plasma and extracted for 1 hour under constant agitation on a thermo-mixer at 37°C. Precipitated proteins were pelleted by centrifugation, and the supernatant transferred to a new tube. 500 µl chloroform was added, followed by 0.2 ml alkaline water to complete the phase separation. The mix was then vortexed and centrifuged at 16,000 x G for 5 minutes. After centrifugation, the upper phase was discarded and the lower organic phase washed 2-3 times with alkaline water (pH 10.3). Finally, the organic phase was dried under N_2 and kept at -20°C until analysis.

Plasma ceramide levels were analyzed by Liquid Chromatography / Mass Spectrometry (LC-MS) as described recently [585]. The ceramide species were separated on a C18 column (Uptispere 120 A, 5 µm, 125 x 2 mm; Interchim, Montlucon, France) and analyzed on a TSQ Quantum Ultra mass spectrometer (Thermo, Reinach, BL, Switzerland). Each sample was measured as a singleton [26]. Intra- and inter-assay coefficient of variation of the method was between 5% and 20%. Analyzed ceramide species included Cer d18:1/14:0, Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/18:1, Cer d18:1/20:0, Cer d18:1/22:0, Cer d18:1/22:1, Cer d18:1/23:0, Cer d18:1/24:0 and Cer d18:1/24:1.

In 19 patients with T1DM the carotid arteries were imaged with a Siemens Sequoia ultrasonography system (Siemens Medical Solutions, Mountain View, CA) with an 8- to 15-MHz linear array transducer. Examination included measurement of common and internal carotid artery flow velocities and Carotid Intima-Media thickness (CIMT) at each of 3 scan planes. Here a mean CIMT of 0.06 cm was considered significant [29].

The clinical and lipid data were merged and clinical baseline characteristics and ceramide levels compared between groups (controls, T1DM, T2DM) by univariate and multivariate linear regression models to control for baseline differences between groups. Normal distribution was tested for all data with the Kolmogorov-Smirnov, D'Agostino and Pearson omnibus and Shapiro-Wilk normality tests. A Bonferroni correction to account for multiple testing in multivariate model building was applied, with a significance level of 0.05. All analyses were performed with SPSS statistical software, version 23.0 (IBM, Zurich, Switzerland). Significance was assessed with Student unpaired, two-tailed t tests or one-way ANOVA.

Results

For this study, we analyzed plasma samples from a total of 153 individuals with T1DM (N = 59), T2DM (N = 40) and healthy volunteers (HV) without diabetes or evidence of metabolic

syndrome (N = 54). Unadjusted demographic and clinical characteristics are shown in Table 9.1.

There were significant differences between the three groups. The T2DM group was on average older (57 +/- 10.8 years) and had higher triglyceride (1.88 +/- 1.30 mmol/l) and lower HDL-C levels (1.25 +/- 0.44 mmol/l) but lower total cholesterol (4.18 +/- 1.12 mmol/l) associated with more prevalent statin use. Previous studies have shown plasma 1-deoxysphingolipids (1-deoxySA and 1-deoxySO) to be significantly increased in patients with dyslipidaemia [560], so univariate and multivariate regression models were used to control for these baseline differences in the lipid profile. Here the healthy volunteers had an apparently more adverse lipid profile than patients with T1DM. No significant difference in HbA1c was seen between patients with T1DM (64 +/- 12 mmol/mol) and T2DM (63 +/- 20 mmol/mol). Twenty-seven of the 59 patients with T1DM were using an insulin pump. The median (and mean) duration of diabetes was 18 years (4-44 years) in patients with T1DM and five patients had been prescribed statin therapy. The median duration of diabetes in patients with T2DM was 13 years (1-39 years). Nineteen of the 40 patients with T2DM were on insulin therapy and thirty-two had been prescribed lipid-lowering medications.

Ceramide levels in the three groups are shown in Table 9.2. The group with T2DM had the lowest levels of all chain lengths of ceramides; levels were highest in the healthy volunteers. LDL cholesterol correlated most strongly with ceramides d18:1/22:0 (R = 0.52, P < 0.001) and d18:1/24:1 (R = 0.50, P < 0.001). LDL cholesterol levels correlated with all the ceramides species analysed however other correlations were weaker (Figure 9.2). HDL cholesterol showed weak correlations with d18:1/14:0, d18:1/16:0, d18:1/22:1 and d18:1/24:0 (Figure 9.3). Triglycerides also showed weak correlations with d18:1/18:0, d18:1/18:1, d18:1/23:0 and d18:1/24:0 (Figure 9.4).

Importantly, patients with type 1 diabetes and evidence of subclinical atherosclerosis on carotid doppler studies had a significantly higher Cer(d18:1/16:0)/Cer(d18:1/24:0) ratio than healthy volunteers (232.44 ± 195.64 *vs.* 142.17 ± 68.29 pmol/ml, P = 0.012), despite a less adverse lipid profile.

Discussion

In this cohort, the ceramide levels were lowest in persons with T2DM and highest in healthy volunteers. This is in contrast with the published literature, which generally indicates that ceramide levels are raised in diabetes, especially given the previous suggestions that ceramides may contribute to insulin resistance as well as pancreatic beta-cell destruction. Interestingly, there were significant correlations between LDL-C and all classes of ceramides suggesting that the lower LDL cholesterol in patients with diabetes may have

affected the results in this study. Statin use was most prevalent in the T2DM group, which also had the lowest levels of ceramides whereas none of the healthy volunteers used any lipid-lowering therapy.

Additionally, statins themselves have been found to reduce ceramide levels and this in itself may have accounted for the paradoxical finding of lower ceramide levels in the T2DM group compared to the other cohorts as the majority of patients in this group were on statins whilst none of the healthy volunteers were. A limitation of this study is that it does not include detailed analysis of type of statin use; previous evidence suggests that within the drug class the influence of statins on ceramide levels is variable. For example rosuvastatin has been shown to decrease ceramide levels in a dose-dependent fashion independent of LDL cholesterol [589]. Given existing clinical guidance on LDL-cholesterol lowering in diabetes, patients in the T2DM cohort were more likely to be using higher potency statins and thus have lower LDL cholesterol levels. The correlation between LDL cholesterol and all ceramide species in this study was at best moderate, so it is likely that there was additional LDL-independent ceramide-lowering within the diabetes groups; most likely due to potent statin use.

One of the controversies surrounding statin use is the development of diabetes. The mechanism by which this occurs is still uncertain as is whether it is simply a class effect of statins as suggested by the meta-analysis by Sattar *et al*. [243], or if potency and dose of statins are likely to have a stronger relationship with diabetes as suggested by another meta-analysis by Preiss and colleagues [241]. The differential effect of statins on ceramide levels therefore appears counterintuitive, as it should offer some protection against the development of diabetes. Less potent statins might therefore be predicted to potentiate any tendency to diabetes. Further research will need to be conducted to determine this with certainty.

This study is the first report of a possible role for plasma ceramides in predicting cardiovascular outcomes in patients with type 1 diabetes. As with recent studies in non-diabetic populations, this variation persists in patients who are already statin-treated, suggesting residual risk.

The assessment and relationship of ceramides with microvascular disease in diabetes was not an objective of this study, however this analysis is currently ongoing in another study, especially with regards to small-fibre neuropathy.

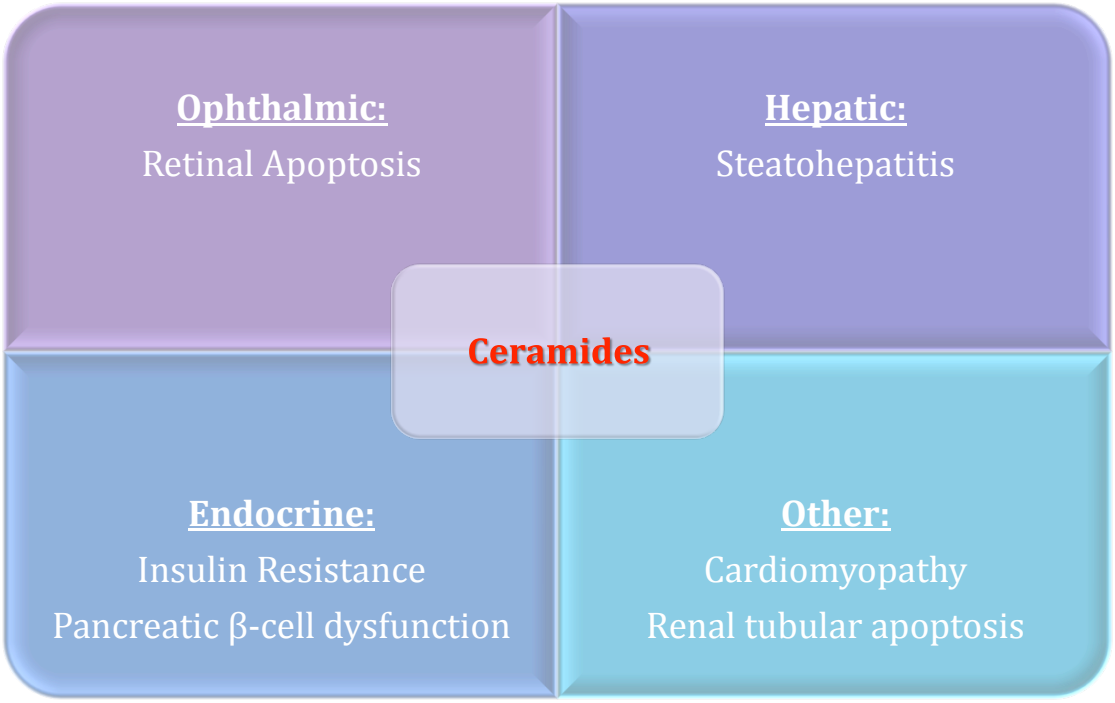

Figure 9.1: The multi-system effects of ceramides

	HV	T1DM	T2DM
Age (years)	41.8 +/- 10.2	39.3 +/- 11.9	57.3 +/- 10.8
Gender (% Male)	33.33	44.07	72.5
BMI (kg/m^2)	25.10 +/- 3.79	25.97 +/- 4.24	31.38 +/- 5.36
Systolic BP (mmHg)	117.8 +/- 15.9	122.9 +/- 15.5	133.4 +/- 17.1
Diastolic BP (mmHg)	73.5 +/- 10.1	76.2 +/- 10.3	72.6 +/- 10.4
Fasting Glucose (mmol/l)	4.53 +/- 0.66	9.19 +/- 4.41	9.17 +/- 5.26
HbA1c (mmol/mol)	-	64.0 +/- 12.0	62.7 +/- 20.4
Total Cholesterol (mmol/l)	5.41 +/- 1.05	4.84 +/- 0.73	4.18 +/- 1.12
Triglycerides (mmol/l)	1.41 +/- 0.83	0.97 +/- 0.40	1.88 +/- 1.30
HDL-C (mmol/l)	1.40 +/- 0.36	1.70 +/- 0.44	1.25 +/- 0.44
LDL-C (mmol/l)	3.36 +/- 0.80	2.72 +/- 0.60	2.06 +/- 0.86

Table 9.1: Demographic and Clinical Characteristics and Results. *HV: Healthy Volunteers; T1DM: Type 1 Diabetes; T2DM: Type 2 Diabetes; BMI: Body Mass Index; BP: Blood Pressure; HbA1c: Glycated Haemoglobin; HDL-C: High-Density Lipoprotein Cholesterol; LDL-C: Low-Density Lipoprotein Cholesterol.* Values shown as mean +/- standard deviation

	HV (µmol/l) N=54	T1DM (µmol/l) N=59	Significance *(HV vs. T1DM)*	T2DM (µmol/l) N=40	Significance *(HV vs. T2DM)*	Significance *(T1DM vs. T2DM)*
Cer d18:1/14:0	285.7 (191-393)	266.0 (194-413)	*NS*	209.9 (119-318)	*NS*	*P=0.04*
Cer d18:1/16:0	2715.7 (2297-2895)	2650.0 (2390-3064)	*NS*	2436.8 (2228-2711)	*NS*	*P=0.04*
Cer d18:1/18:1	234.0± 75.9	200.9± 47.0	*P=0.016*	207.7± 53.7	*P=0.023*	*NS*
Cer d18:1/18:0	379.3± 106.4	342.0± 71.5	*NS*	353.6± 79.3	*NS*	*NS*
Cer d18:1/20:0	618.4± 150.6	585.2± 153.1	*NS*	553.5± 157.5	*P=0.034*	*NS*
Cer d18:1/22:1	136.4 (90-183)	134.9 (75-144)	*NS*	110.9 (75-144)	*NS*	*NS*
Cer d18:1/22:0	1390.6± 330.8	1282.9± 351.7	*NS*	1180.2± 392.9	*P=0.041*	*P=0.047*
Cer d18:1/24:1	2603.0± 576.0	2498.7± 641.5	*NS*	2170.7± 726.6	*P=0.006*	*P=0.047*
Cer d18:1/23:0	46.3± 14.3	43.4± 14.0	*NS*	42.2± 15.5	*NS*	*NS*
Cer d18:1/24:0	210.2± 66.5	197.9± 66.0	*NS*	196.6± 82.7	*NS*	*NS*

Table 9.2: Ceramide levels in different cohorts. *HV: Healthy Volunteers; T1DM: Type 1 Diabetes; T2DM: Type 2 Diabetes; Cer: Ceramide. NS: non-significant.*

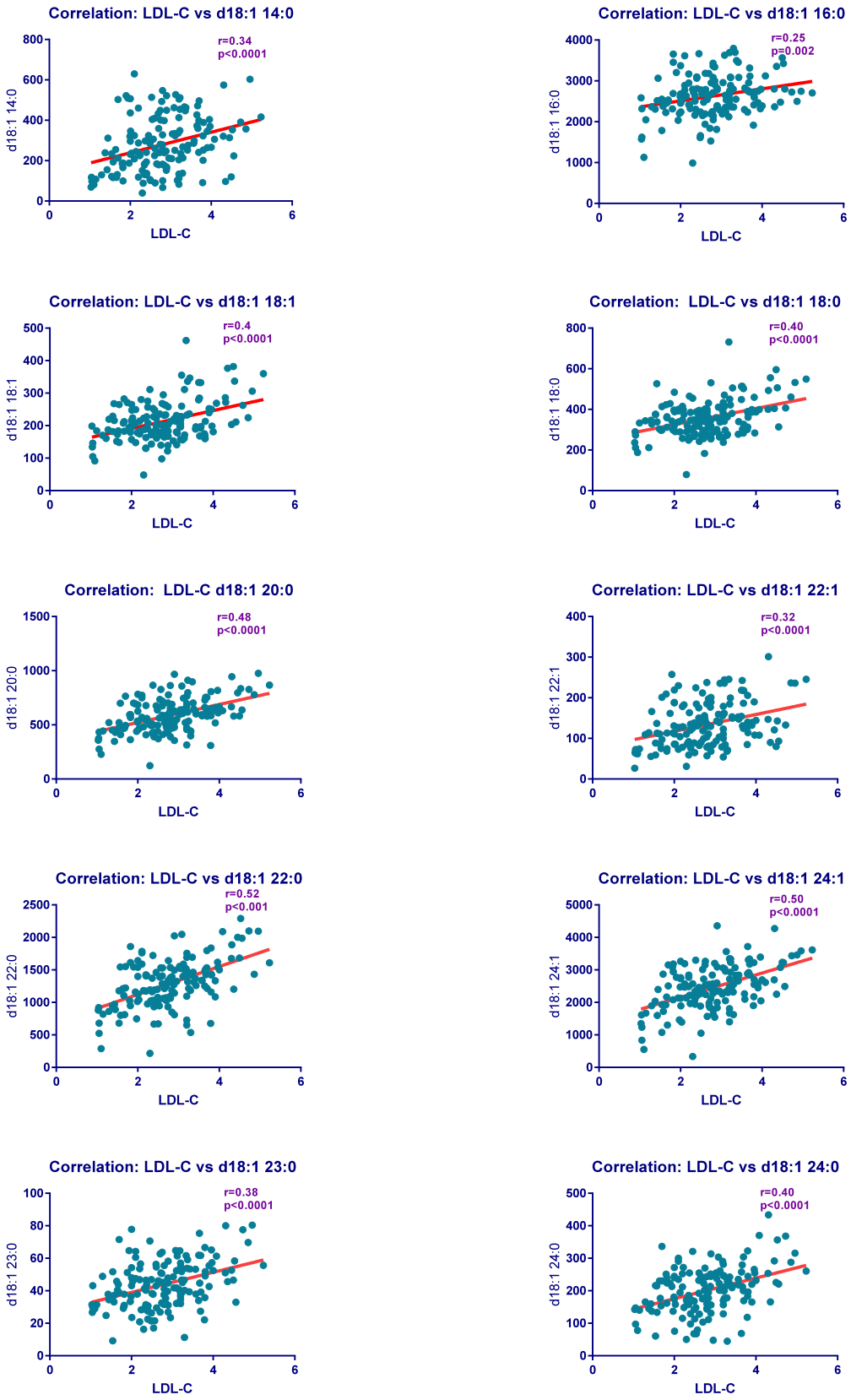

Figure 9.2: LDL correlations with different ceramide species. *LDL-C: LDL cholesterol (mmol/l)*

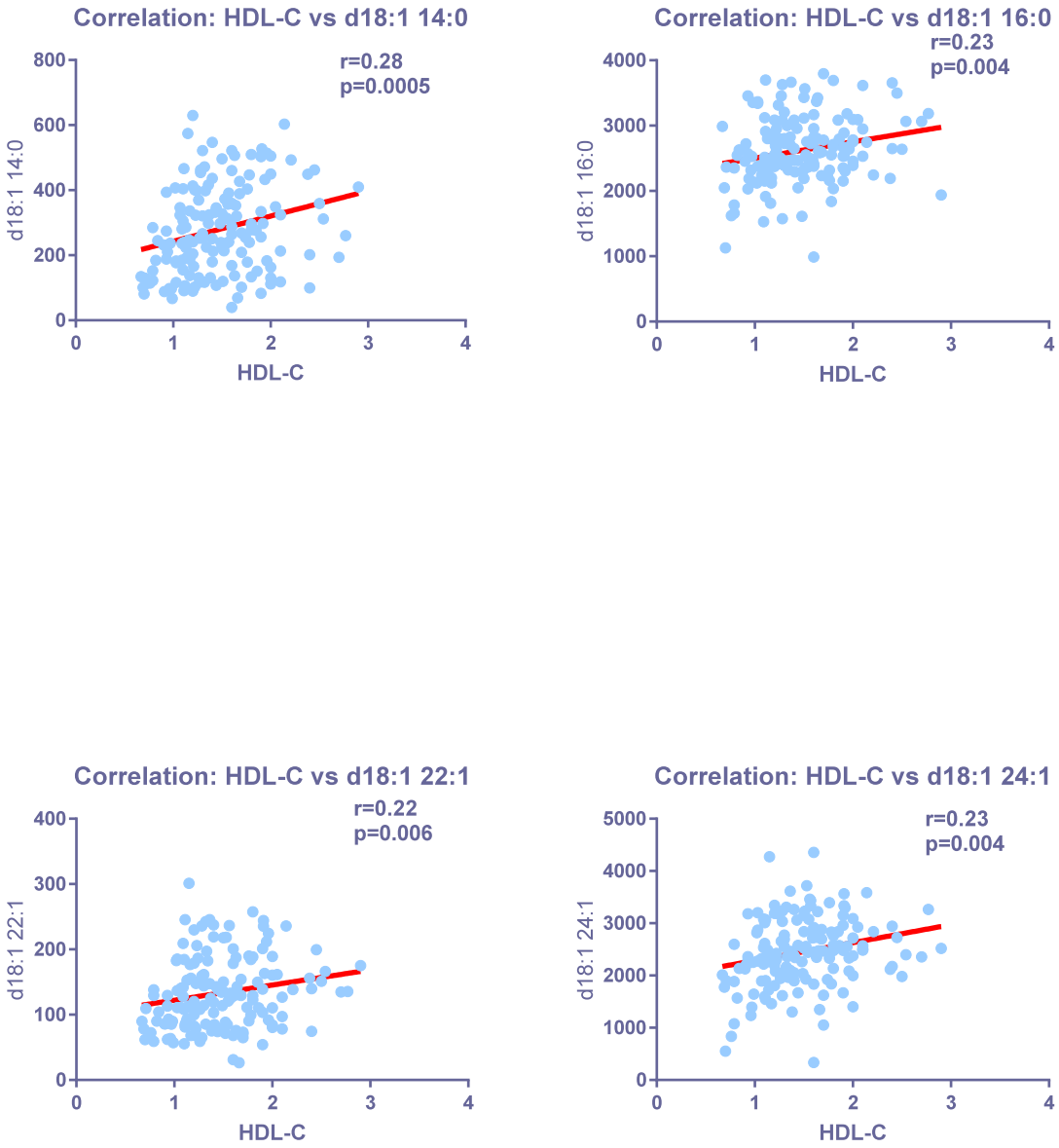

Figure 9.3: HDL correlations with different ceramide species. *HDL-C: HDL cholesterol (mmol/l)*

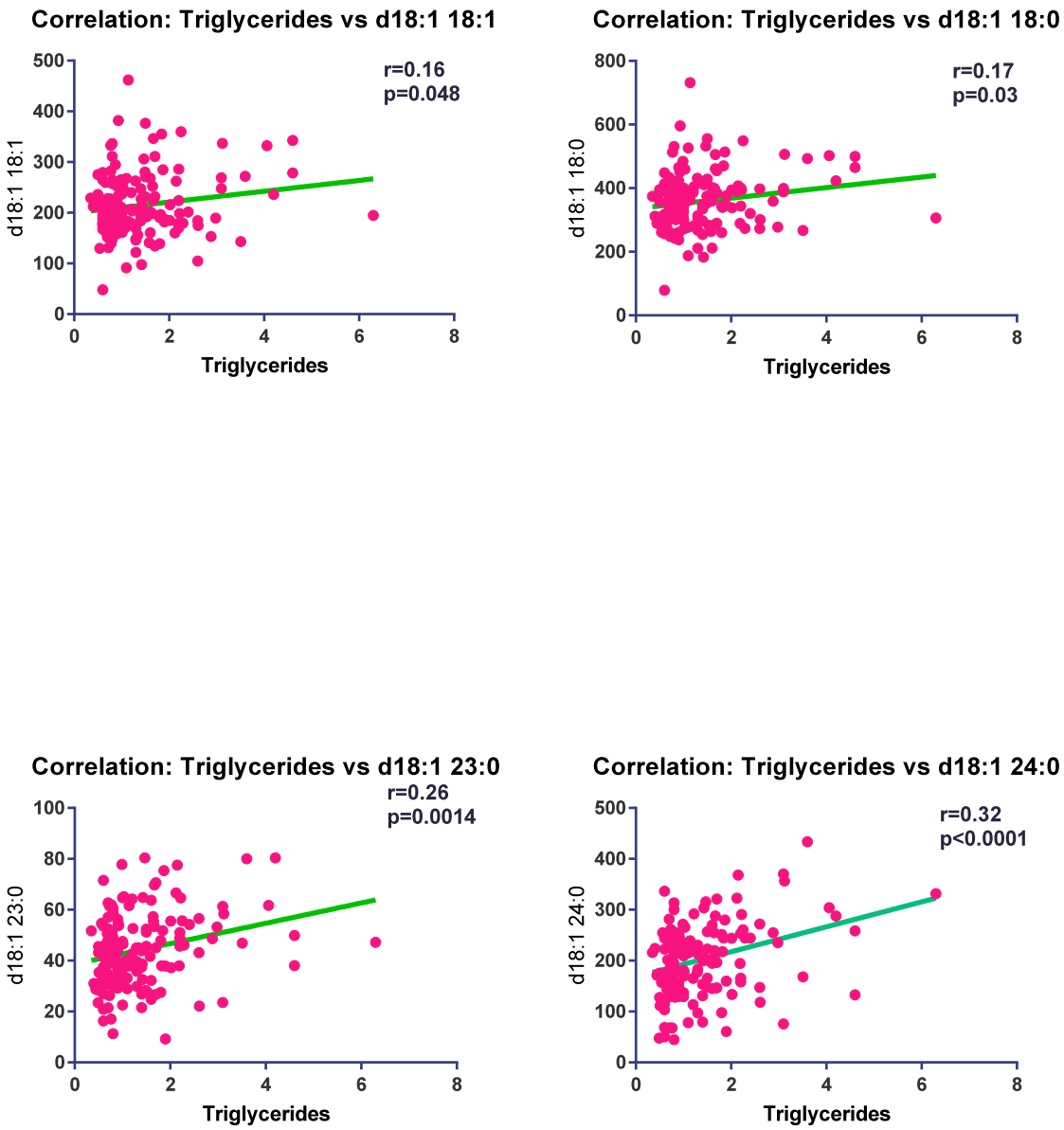

Figure 9.4: Triglyceride correlations with different ceramide species

Conclusion

Although the inverse association between high-density lipoprotein (HDL) levels and cardiovascular disease (CVD) risk is well established [39, 40], and not affected by statin therapy [47], the 'HDL hypothesis', that therapeutic interventions directed at raising HDL cholesterol might translate into improved cardiovascular outcomes currently hangs by a thread. The expectation that modification of HDL levels might reduce CVD risk through its pleiotropic effects seems confounded by recent reports from genetic and pharmacological studies [9].

The mechanism by which HDL-cholesterol is increased is critical in determining whether it reduces atherosclerosis. Although HDL cholesterol levels are a strong biomarker for assessing CVD risk, they predict neither HDL functionality or composition [10]. HDL can undergo modification in structure and composition to become dysfunctional in conditions associated with systemic inflammation and oxidative stress [72, 73].

Fenofibrate, a peroxisome proliferator-activated receptor-α (PPAR-α) agonist, is often used in the management of dyslipidaemia, principally for its triglyceride lowering and HDL cholesterol raising effects. Paradoxical reductions in HDL cholesterol levels have been reported on treatment. The molecular mechanisms for such paradoxical reductions and their impact on cardiovascular risk have not previously been studied. My observation that paradoxical reductions in HDL cholesterol are accompanied by a parallel decrease in apolipoprotein AI (apo AI) suggests underlying decreased synthesis or increased catabolism. Decreased apo AI levels are accompanied by reduced cholesterol efflux capacity, and are associated with increased cardiovascular risk.

Cardiovascular disease is the leading cause of death and disability in diabetes. Patients with diabetes show qualitative and kinetic lipoprotein abnormalities [191], and any cardiovascular benefit associated with intensive glucose lowering may be related to effects on lipoprotein metabolism rather than directly through altered glycaemia [188].

The apparently relatively undisturbed lipid profile observed in many patients with diabetes may hide major atherogenic changes [18-20]. Low-density lipoprotein (LDL) is the permissive factor in the development of atherosclerosis but must undergo oxidation and / or glycation to participate in atherogenesis. The relationship between oxidation and glycation is complex; this work explores factors facilitating apolipoprotein B (apo B) glycation and oxidation, including the role of transition metal ions [207, 208], and in particular interactions of copper with apo B and how these might be attenuated through copper-selective chelation. I have also demonstrated the increased prevalence of atherogenic LDL in Type 1 Diabetes (T1DM), its contribution to cardiovascular outcomes,

and how HDL may be dysfunctional in its capacity to protect LDL against these atherogenic modifications.

LDL cholesterol remains the primary focus in lipid modification for the prevention and treatment of atherosclerosis [17]. This thesis presents data to support more aggressive management of LDL cholesterol levels than currently advised. The relative risk reduction achieved with statin treatment is similar in patients with diabetes to that in other people. However, the number needed to treat to prevent one event (NNT) will be lower in patients with diabetes compared to those without diabetes but apparently similar lipid profiles [21].

This thesis also demonstrates that the current use of derived lipid profile measurements including LDL, non-HDL cholesterol and remnant cholesterol rather than apo B (and apo AI) measurements may also offer false reassurance and exacerbate under-treatment of cardiovascular risk. Recent work suggests that much of the excess CVD incidence in diabetes is because of inadequate treatment of lipids and BP [269]. Recent reports suggest that cardiovascular mortality in young women with T1DM now exceeds that of men [270]; these young women are the patient group in whom statins are most often withheld [271].

HDL cholesterol levels are normal or even high in T1DM prior to the development of nephropathy, but do not seem to protect against atherosclerosis as might be expected. This thesis offers new insight into HDL functionality in T1DM, exploring effects on reverse cholesterol transport, HDL anti-oxidant, anti-glycative, and anti-inflammatory functions, and HDL-associated enzymes, including paraoxonase-1. The findings reported here raise the possibility that a substantial portion of the protective effect of HDL may be due to functions beyond reverse cholesterol transport [12]. HDL dysfunction should also be considered when reviewing the lipid profile and derived measurements in patients with T1DM. I have also explored the contribution of HDL dysfunction to microvascular diabetic complications, in particular neuropathy, alongside consideration of sphingolipids and deoxysphingolipids as novel biomarkers for neuropathy.

This thesis also considers alterations in markers of inflammation and endothelial dysfunction in T1DM, including novel cardiovascular risk biomarkers, alongside measures of subclinical atherosclerosis, which might also lead to the integration of these biomarkers into risk prediction models. This work compares 1-deoxysphingolipid levels between individuals with T1DM, type 2 diabetes (T2DM) and healthy controls. Sphingolipid metabolism and the contribution of 1-deoxysphingolipids to T1DM and its complications would appear to be more complex than the interactions recently reported in T2DM. This thesis includes the first report of a possible role for plasma ceramides in predicting cardiovascular outcomes in patients with T1DM.

In summary, lipoprotein metabolism in T1DM remains relatively poorly researched. This work offers new insight, reporting atherogenic changes in apo B-containing lipoproteins, apolipoprotein distribution, ceramides, sphingolipids and HDL functionality.

Future work based on this thesis will include assessments of lipoprotein qualitative changes in clinical trials of the selective copper-chelator triethylenetetramine (TETA), further assessments of HDL functionality, and examination of HDL particle distribution in T1DM. I am also keen to explore postprandial changes in HDL functionality and lipoprotein quality, which may be more marked in T1DM than other conditions associated with increased cardiovascular risk.

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