LIPOPROTEIN MARKERS OF CARDIOVASCULAR RISK IN METABOLIC CONDITIONS AND THE IMPACT OF INTERVENTIONS

A thesis submitted to the University of Manchester for the degree of

Doctor of Philosophy

in the Faculty of Biology, Medicine and Health

2020

Jan Hoong Ho

School of Medical Sciences

Division of Cardiovascular Sciences

LIST OF CONTENTS

Word Count: 58 688

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

-C	Cholesterol
ABC-	ATP-binding cassette transporter
AES	Androgen Excess Society
AIM-HIGH	Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes
ALT	Alanine transaminase
Apo(a)	Apolipoprotein(a)
ApoA-I	Apolipoprotein A-I
ApoB	Apolipoprotein B-100
ApoB-IC	Apolipoprotein B-100 immune complex
ASCVD	Atherosclerotic cardiovascular disease
AST	Aspartate transaminase
BMI	Body mass index
BSA	Bovine serum albumin
C-AMP	8-(4-chlorophenylthio) adenosine 3’,5’-cyclic monophosphate sodium salt
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CHOD-PAP	Cholesterol oxidase phenol 4-aminoantipyrine peroxidase
CI	Confidence interval
CNBr	Cyanogen bromide
CRP	C-reactive protein
CV	Coefficient of variation

CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHEAS	Dehydroepiandrosterone sulphate
eGFR	Estimated glomerular filtration rate
EPIC-Norfolk	European Prospective Investigation into Cancer Norfolk Cohort
ERN	Extended release niacin
ERN/LRP	Extended release niacin with laropiprant
ESHRE/ASRM	European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine
GGT	Gamma glutamyl transferase
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GPO-PAP	Glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase
HbA1c	Glycosylated haemoglobin
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HOMA-B	Homeostatic model assessment of beta cell function
HOMA-IR	Homeostatic model assessment of insulin resistance
HPS2-THRIVE	Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IC	Immune complex
ICAM-1	Intercellular adhesion molecule-1

IL-6	Interleukin-6
K	Kringle
LDL	Low-density lipoprotein
Lp(a)	Lipoprotein(a)
LpPLA2	Lipoprotein-associated phospholipase A2
MCP-1	Monocyte chemotactic protein-1
MDA-LDL	Malondialdehyde-modified low-density lipoprotein
mRNA	Messenger ribonucleic acid
miRNA	Micro-ribonucleic acid
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NICE	National Institute of Health and Care Excellence
NIH	National Institute of Health
nM	Nanomolar
NO	Nitric oxide
OD	Optical density
OSE	Oxidation-specific epitopes
OxLDL	Oxidised low-density lipoprotein
OxPL	Oxidised phospholipids
OxPL-apo(a)	Oxidised phospholipids on apolipoprotein(a)
OxPL-apoB	Oxidised phospholipids on apolipoprotein B-100 containing lipoproteins

PAH	Platelet-activity factor acetylhydrolase
PBS	Phosphate buffer solution
PC	Phosphocholine
PCOS	Polycystic ovarian syndrome
PCSK9	Proprotein convertase subtilisin/kexin 9
PEG	Polyethylene glycol
PON1	Paraoxonase-1
PREVEND	Prevention of Renal and Vascular End-stage Disease
PVD	Peripheral vascular disease
REC	Research ethics committee
RLU	Reactive light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RQV	Relative quantitative value
RYGB	Roux-en-Y gastric bypass
SAA	Serum amyloid A
SBP	Systolic blood pressure
sdLDL	Small dense low-density lipoprotein
SHBG	Sex hormone binding globulin
SR-B1	Scavenger receptor class B type 1
TBS	Tris-buffered saline
TMB	3,3’,5,5’-tetramethylbenzidine
TNFα	Tumour necrosis factor alpha

UCSD	University of California San Diego
VCAM-1	Vascular adhesion molecule-1
VLDL	Very low-density lipoprotein
WHO	World Health Organization

ABSTRACT

Atherogenic modification of low-density lipoprotein (LDL) and impaired high-density lipoprotein (HDL) functionality are key contributors to atherosclerosis and cardiovascular disease (CVD). Markers of LDL modification and HDL functionality are therefore important biomarkers of CVD risk that could help provide insights into the underlying pathophysiology and the impact of risk modification therapies. Oxidative modification of LDL results in generation of various oxidation specific epitopes, including oxidised phospholipids (OxPL) which are preferentially bound to lipoprotein(a) [Lp(a)] in circulation. OxPL measured on apolipoprotein B-100 (OxPL-apoB) is closely associated with Lp(a) and both are independent predictors for CVD. HDL has multiple atheroprotective functions including cholesterol efflux capacity, and more recently, it has emerged as a transporter of micro-ribonucleic acids (miRNA) which have been shown to confer some of the protective effects of HDL.

This thesis examined these lipoprotein markers of CVD risk in obesity and the changes that occur after bariatric surgery, as well as in polycystic ovarian syndrome (PCOS) with specific focus on the impact of insulin resistance. The effects of extended release niacin and laropiprant (ERN/LRP) added to statin therapy on levels of OxPL-apoB and Lp(a) were also assessed.

Studies in this thesis demonstrated a divergence in OxPL-apoB and Lp(a) following bariatric surgery which was not previously reported. The decrease in OxPL-apoB reflect an overall reduction in oxidative stress while the increase in Lp(a) was postulated to relate to improvement in hepatic steatosis and function resulting in increased hepatic synthesis. Expression of specific HDL-associated miRNAs were increased after bariatric surgery, in association with improvements in other markers of HDL functionality, indicating an overall improvement in HDL function. Insulin resistance was shown to be a key factor underlying the CVD risk associated with PCOS, which was reflected by impairment in HDL functionality and markers of oxidative modification and glycation of LDL. Levels of OxPL-apoB and Lp(a) can be impacted by lipid-modifying therapies, and reduction in Lp(a) but not OxPL-apoB was observed with ERN/LRP when added to statin therapy.

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 or other institute of learning.

COPYRIGHT STATEMENT

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/about/regulations/) and in The University’s policy on Presentation of Theses.

To Jane,

for the love and patience throughout this wonderful journey together,

and to Wei Ann and Wei Yi,

for the joy of watching you grow.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my main supervisor Dr Handrean Soran for this research opportunity, which has provided me with a foundation to further my career, and also allowed me to develop at a personal level. I am forever grateful for his continuous encouragement and inspiration throughout the duration of my PhD work.

I would like to thank Dr Rachelle Donn for providing me with support and guidance, and Professor Rayaz Malik for the invaluable feedback and wisdom on conducting research studies.

A special thank you to Dr Safwaan Adam and Dr Shaishav Dhage for the intellectual discussions and the friendship that have developed through working together and supporting each other. I would also like to thank Dr Yifen Liu for being an excellent and dedicated teacher in the laboratory, constantly providing me with help and guidance, without which my research work would not have been possible, and to Dr Maryam Ferdousi and Dr Alise Kalteniece, for the useful discussions and different perspectives during team meetings.

I am also grateful to the weight management team at Salford Royal NHS Foundation Trust, in particular, Dr Akheel Syed and Professor Basil Ammori, who have provided invaluable guidance and support during patient recruitment. Jodi Ellison and Chris Slater have also been extremely supportive throughout the patient recruitment process, going above and beyond their clinical duties.

I would also like to extend my gratitude to Professor Sotirios Tsimikas at the University of California San Diego for providing guidance on the oxidised phospholipid work, and to Professor Kerry-Anne Rye and Dr Fatiha Tabet at the University of New South Wales for being extremely accommodating and supportive during my visit to their research laboratory which facilitated the work on HDL-associated miRNA.

To my parents, without whom nothing in life would have been possible. No words can ever be enough to express how much I owe them for the unconditional love and support since childhood, and the incredible amount of sacrifice and burden they have shouldered, which have allowed me to pursue a better future.

Finally, I would also like to acknowledge and thank all the research participants who have volunteered their time and research staff at the Cardiovascular Trials unit and the NIHR/Wellcome Trust Clinical Research Facility at Manchester University NHS Foundation Trust.

CONTRIBUTIONS

The author of this thesis has contributed significantly to all of the chapters and studies presented in this thesis. He co-authored the study protocols and study visit documents for the bariatric surgery study and presented to the Research Ethics Committee. The author led and undertook the patient recruitment, consent process, study visit assessments, and processing of blood samples for storage, and database management for the bariatric surgery study. He undertook laboratory analyses for assays performed at the University of Manchester lipid research laboratory with the help and supervision of Dr Yifen Liu, apart from the extended-release niacin and laropiprant study. He coordinated transfer of samples to international collaborators (Professor Sotirios Tsimikas, Professor Nicolas Vuilleumier, and Professor James Gibney). The author undertook data analyses and interpretation for all the results chapters presented in this study.

Contributions from other members of the research team and international collaborators:

Dr Safwaan Adam undertook some of the patient recruitment, study visit assessments, and processing of blood samples.

Dr Shaishav Dhage undertook some of the study visit assessments.

All the laboratory analyses by the author were undertaken together with Dr Yifen Liu.

Measurements of oxidised phospholipids and lipoprotein(a) were undertaken at the University of California San Diego under Professor Sotirios Tsimikas.

Measurements of anti-apoA-I IgG was undertaken by Dr Sabrina Pagano in the laboratory of Professor Nicolas Vuilleumier.

Data analyses for chapter 5 was undertaken together with Dr Kwok Leung Ong with guidance from Dr Fatiha Tabet.

JOURNAL FORMAT THESIS

The author has been granted permission by his main supervisor Dr Handrean Soran, and co-supervisors Dr Rachelle Donn and Professor Rayaz Malik to submit this PhD thesis in journal format under the regulations of the University of Manchester, Faculty of Biology, Medicine and Health, including sections which are in a format suitable for submission for publication or dissemination. This format facilitates dissemination and publication, and will therefore increase the impact of the research work completed. The following chapters in this thesis have been submitted or are being submitted for publication:

Chapter 4: Submitted to Journal of Clinical Lipidology

Chapter 5: Submitted to Journal of Lipid Research

Chapter 6: To be submitted for publication

Chapter 7: To be submitted for publication

PREFACE

Jan Hoong Ho completed his undergraduate medical training at the University of Manchester in 2009 after undertaking pre-clinical training at the International Medical University in Malaysia. He completed his foundation and core medical training in the north west of England, during which he was awarded his MRCP in 2012. He then obtained his National Training Number in Diabetes, Endocrinology and General Internal Medicine in 2013. He completed his Speciality Certificate Examination in Diabetes & Endocrinology in 2015. Between 2016 and 2019, he took 3 years out of his speciality training to undertake this PhD at the University of Manchester. He is currently in his final year of specialty training which is due to complete in January 2021.

He has always demonstrated a passion for Diabetes & Endocrinology, and have contributed to the Society for Endocrinology annual BES conferences with poster presentations since foundation training. It was during his rotation at the Manchester Royal Infirmary where he was regular involved in the tertiary lipid clinic that he developed his interest in the management cardiovascular risk in metabolic conditions, leading to undertaking his research in this field.

During his time in research, he has published 18 peer-reviewed publications and has delivered oral presentations on aspects of his research at prestigious national and international meetings including the Royal Society of Medicine bariatric surgery and lipidology update (2017), British Obesity and Metabolic Surgery Society annual conference (2018), European Atherosclerosis Society annual congress (2018 and 2019), and HEART UK annual conference (2019). A number of the research work that he undertook was awarded prizes for best oral communication at national conferences including the British Obesity and Metabolic Surgery Society annual conference (2018) and the Association of British Clinical Diabetologists annual SpR meeting (2018), and outstanding abstract and nomination for President's Prize at the Endocrine Society annual ENDO meeting (2019). He has also been an invited speaker and chaired sessions at the HEART UK annual conference (2019) and North West Lipid Forum (2020).

In 2018, he was awarded the prestigious Royal College of Physician Samuel Leonard Simpson travelling fellowship in endocrinology, which funded his visit to Prof Rye's research laboratory at University of New South Wales Sydney, where he learnt and brought the method for assessing HDL-mediated glucose-stimulated insulin secretion in pancreatic beta cells back to the lipid research laboratory at the University of Manchester.

LIST OF PUBLICATIONS & ABSTRACTS

List of publications during period of registration:

1. Iqbal Z, **Ho JH**, Adam S, France M, Syed A *et al*. Managing hyperlipidaemia in patients with COVID-19 and during its pandemic: An expert panel position statement from HEART UK. Atherosclerosis. 2020 Sep 15. doi: 10.1016/j.atherosclerosis.2020.09.008 [Epub ahead of print].
2. Iqbal Z, Adam S, **Ho JH**, Syed AA, Ammori BJ, Malik RA, Soran H. Metabolic and cardiovascular outcomes of bariatric surgery. Curr Opin Lipidol. 2020 Aug;31(4):246-256. doi: 10.1097/MOL.0000000000000696.
3. Soran H, France M, Adam S, Iqbal Z, **Ho JH** *et al*. Quantitative evaluation of statin effectiveness versus intolerance and strategies for management of intolerance. Atherosclerosis. 2020 Aug;30(6):33-40. doi: 10.1016/j.atherosclerosis.2020.06.023. Epub 2020 Jul 6.
4. Kwok S, Adam S, **Ho JH**, Iqbal Z, Turkington P *et al*. Obesity: A critical risk factor in the COVID-19 pandemic. Clin Obes. 2020 Aug 28:e12403. doi: 10.1111/cob.12403. Online ahead of print.
5. Dhage S, **Ho JH**, Ferdousi M, Kalteniece A, Azmi S *et al*. Small fibre pathology is associated with erectile dysfunction in men with type 2 diabetes. Diabetes Metab Res Rev. 2020 Mar;36(3):e3263. doi: 10.1002/dmrr.3263. Epub 2019 Dec 18.
6. Iqbal Z, Dhage S, Mohamad JB, Abdel-Razik A, Donn R, Malik R, **Ho JH** *et al*. Efficacy and safety of PCSK9 monoclonal antibodies. Expert Opin Drug Saf. 2019 Dec;18(12):1191-1201. doi: 10.1080/14740338.2019.1681395. Epub 2019 Nov 20.
7. Adam S, Liu Y, Siahmansur T, **Ho JH**, Dhage SS *et al*. Bariatric surgery as a model to explore the basis and consequences of the Reaven hypothesis: Small, dense low-density lipoprotein and interleukin-6. Diab Vasc Dis Res. 2019 Mar;16(2):144-152. doi: 10.1177/1479164119826479.
8. Soran H, Adam S, **Ho JH**, Durrington PN. The Reaven syndrome: An historical perspective. Diab Vasc Dis Res. 2019 Mar;16(2):116-117. doi: 10.1177/1479164119828899.
9. **Ho JH**, Adam S, Azmi S, Ferdousi M, Liu Y, *et al.* (2019) Male sexual dysfunction in obesity: The role of sex hormones and small fibre neuropathy. PLoS

One. 2019 Sep 11;14(9):e0221992. doi: 10.1371/journal.pone.0221992. eCollection 2019.

10. Soran H, **Ho JH**, Adam S, Durrington PN. Non-HDL cholesterol should not generally replace LDL cholesterol in the management of hyperlipidaemia. Curr Opin Lipidol. 2019 Aug;30(4):263-272. doi: 10.1097/MOL.0000000000000614.

11. Schofield J, **Ho JH**, Soran H. Cardiovascular risk in type 1 diabetes. Diabetes Ther. 2019 Jun;10(3):773-789. doi: 10.1007/s13300-019-0612-8. Epub 2019 Apr 19.

12. Soran H, **Ho JH**, Durrington PN. Acquired low cholesterol: diagnosis and relevance to safety of low LDL therapeutic targets. Curr Opin Lipidol. 2018 Aug;29(4):318-326. doi: 10.1097/MOL.0000000000000526.

13. Soran H, Banerjee M, Mohamad JB, Adam S, **Ho JH** *et* al. Risk Factors for Failure of Direct Current Cardioversion in Patients with Type 2 Diabetes Mellitus and Atrial Fibrillation. Biomed Res Int. 2018 Mar 12;2018:5936180. doi:10.1155/2018/5936180. eCollection 2018

14. Soran H, Adam S, Mohammad J, **Ho JH**, Schofield J *et al.* Hypercholesterolaemia – practical information for non-specialists. Arch Med Sci. 2018 Jan;14(1):1-21. doi: 10.5114/aoms.2018.72238. Epub 2017 Dec 19.

15. Soran H, Liu Y, Adam S, Siahmansur T, **Ho JH** *et al.* A comparison of the effects of low- and high-dose atorvastatin on lipoprotein metabolism and inflammatory cytokines in type 2 diabetes: Results from the Protection Against Nephropathy in Diabetes with Atorvastatin (PANDA) randomized trial. J Clin Lipidol. 2018 Jan-Feb;12(1):44-55. doi: 10.1016/j.jacl.2017.10.011. Epub 2017 Nov 13.

16. Yadav R, Hama S, Liu Y, Siahmansur T, Schofield J, Syed AA, France M, Pemberton P, Adam S, **Ho JH** *et al.* Effect of Roux-en-Y bariatric surgery on lipoproteins, insulin resistance, and systemic and vascular inflammation in obesity and diabetes. Front Immunol. 2017 Nov 15;8:1512. doi: 10.3389/fimmu.2017.01512. eCollection 2017.

17. Soran H, Kwok S, Adam S, **Ho JH**, Durrington PN. Evidence for more intensive cholesterol lowering. Current opinion in lipidology. 2017 Aug. Available from, DOI:10.1097/MOL.0000000000000433

18. France M, Kwok S, Soran H, Williams S, **Ho JH** *et al.* Liver fat measured by MR spectroscopy: Estimate of imprecision and relationship with serum glycerol, caeruloplasmin and non-esterified fatty acids. Int J Mol Sci. 2016 Jul 8;17(7):1089. doi: 10.3390/ijms17071089.

List of conference presentations and abstracts during period of registration:

1. **Ho JH**, Liu Y, Adam S, Azmi S, Dhage SS *et al*. The effect of metabolic surgery on lipoprotein(a), oxidised phospholipids and biomarkers of lipoprotein oxidation. (Oral Presentation, HEARTUK Annual Conference, Warwick, 2019)
2. Adam S, Azmi S, Liu Y, Ferdousi M, Siahmansur T, Kalteniece A, Marshall A, **Ho JH** *et al*. Changes in serum triglycerides are associated with improvements in small fibre neuropathy in obese persons following bariatric surgery. (Oral Communication, HEARTUK Annual Conference, Warwick, 2019)
3. Liu Y, Dhage S, France F, Adam S, **Ho JH** *et al*. Variation and distribution of apolipoprotein E and its glycation in plasma of type 2 diabetes. (Poster Presentation at 87th EAS Congress, Maastricht, 2019)
4. **Ho JH**, Adam S, Liu Y, Azmi S, Dhage SS *et al*. Reduction in autoantibodies to oxidised LDL and apoB immune complexes following metabolic surgery (Moderated Poster Presentation at 87th EAS Congress, Maastricht, 2019)
5. **Ho JH**, Liu Y, Adam S, Azmi S, Dhage SS *et al*. Divergent changes in lipoprotein(a) and oxidised phospholipids following metabolic surgery. (Poster Presentation at 87th EAS Congress, Maastricht, 2019)
6. Garside B, Liu Y, Neelamekam S, **Ho JH**, Dhage SS *et al*. Apolipoprotein B-100 decrease in association with reduction in PCSK9 concentration in Acid Sphigomyelinase Deficiency following enzyme replacement therapy. (Moderated Poster Presentation at 87th EAS Congress, Maastricht, 2019)
7. Adam S, Minder Steimer AE, **Ho JH**, Whittle L *et al*. The health burden of adrenal insufficiency in a United Kingdom-based population. (Featured Poster Presentation at ENDO 2019 [*awarded Outstanding Abstract Award* and nomination of President's Prize])
8. Dhage SS, **Ho JH**, Ferdousi M, Kalteniece A, Azmi S *et al*. Small not large fibre pathology is associated with erectile dysfunction in men with type 2 diabetes. (Poster Presentation at Diabetes UK Annual Meeting, Liverpool, 2019)
9. Adam S, T Siahmansur T, Liu Y, **Ho JH**, Pagano S *et al*. Bariatric surgery leads to a reduction in anti-apolipoprotein-A-1 IgG antibodies. (Poster Presentation at 86th EAS Congress, Lisbon, 2018)

10. Dhage SS, Azmi S, Adam S, Ferdousi M, Liu Y, Siahmansur TJ, Ponirakis G, Marshall A, Alam U, Petropoulos I, Pemberton P, Schofield J, **Ho JH** *et al*. Obesity related neuropathy is associated with HDL functionality. (Poster Presentation at 86th EAS Congress, Lisbon, 2018)

11. **Ho JH**, Adam S, Siahmansur T, Liu Y, Azmi S *et al*. Bariatric surgery enhances reverse cholesterol transport and high-density lipoprotein functionality. (Moderated Poster Presentation at 86th EAS Congress, Lisbon, 2018)

12. Liu Y, Adam S, **Ho JH**, Siahmansur T, Azmi S *et al*. Reductions in glycated apolipoprotein B after bariatric surgery are more closely associated with decreases in glycaemic parameters than changes in LDL-C or total apolipoprotein B. (Moderated Poster Presentation at 86th EAS Congress, Lisbon, 2018)

13. Adam S, Liu Y, Siahmansur T, **Ho JH**, Azmi S *et al*. Novel insights into potential mechanisms by which bariatric surgery reduces cardiovascular disease risk. (Oral Presentation at British Obesity and Metabolic Surgery Society Annual Meeting, Telford, 2018) [*awarded Best Oral Communication Award*].

14. **Ho JH**, Adam S, Azmi S, Dhage SS, Liu Y *et al*. The effects of bariatric surgery on obesity-related male sexual dysfunction. (Oral Presentation at British Obesity and Metabolic Surgery Society Annual Meeting, Telford, 2018)

15. **Ho JH**, Adam S, Azmi S, Dhage S, Liu Y *et al.* Male sexual dysfunction and hypogonadism improves following bariatric surgery. (Poster Presentation, Society for Endocrinology BES Annual Conference, Harrogate, 2017).

16. Dhage SS, Adam S, **Ho JH**, Yadav R, Hama S *et al.* Effect of Roux-en-Y bariatric surgery on lipoproteins, insulin resistance, and systemic and vascular inflammation in obese patients with and without diabetes. (Poster Presentation, SfE BES, Harrogate, 2017).

CHAPTER 1: INTRODUCTION

1.1 CARDIOVASCULAR DISEASE: DEFINITION AND EPIDEMIOLOGY

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, with an estimated 17.9 million deaths annually, accounting for approximately one third of all mortality globally.[1] The World Health Organization (WHO) defines CVD to include coronary heart disease (CHD), cerebrovascular disease, peripheral vascular disease (PVD), hypertension, heart failure, rheumatic heart disease, congenital heart disease, and cardiomyopathies.[1] Atherosclerotic CVD (ASCVD) comprises mainly of CHD, cerebrovascular disease, and PVD; and accounts for the majority of CVD mortality.[1]

CVD is more prevalent among the developing world, and although there has been a recent decline in age-standardised mortality and premature mortality in the UK,[2] it remains a close second in cause of death behind cancer, and the burden of disease extends beyond the mortality figures.[3] Despite the decline in mortality, the prevalence of CVD in the UK is a relatively constant trend, based on data from the Quality and Outcomes Framework, and numbers for CVD-related hospital admissions and prescriptions, and percutaneous coronary interventions have all increased markedly reflecting the rising health burden of CVD. The annual costs attributed to CVD are estimated at £7.4 billion in direct healthcare cost and £15.8 billion to the wider economy.[4]

Mortality and morbidity statistics show evidence of wide inequalities across Europe, with similar trends of declining CVD mortality in Western Europe, but much higher rates in Eastern Europe.[5] Similar to the UK, there were increasing rates of hospitalisation and transluminal coronary angioplasties across most countries,[5] and high disability-adjusted life years of over 150 per 1000 population in a number of countries amid a wide range reported across Europe.[6] In the United States, despite mortality rates trending downwards over the past decade, CVD remains the leading cause of death, with mortality rates exceeding cancer and lung disease combined. It is estimated that close to half the population in the United States will have some form of CVD leading to an estimated total cost of $1.1 trillion by 2035.[7]

1.2 ATHEROSCLEROTIC CARDIOVASCULAR DISEASE: PATHOPHYSIOLOGY

Although the CVD umbrella covers a wide range of disorders affecting the heart and vasculature, ASCVD is the predominant contributor of mortality and morbidity,[1, 3] making atherosclerosis the most important underlying pathological process in CVD.

The understanding of the pathophysiology of ASCVD has developed and evolved significantly over the past few decades, and is now established as a pathological inflammatory process in the arteries driven by lipid, metabolic and immunological alterations.[8, 9]

1.2.1 INFLAMMATION

Atherosclerosis is a lipid-driven inflammatory process affecting the intima of the arterial wall.[10] It had been historically considered a disease of lipid accumulation until the role for inflammation came to prominence towards the end of the twentieth century.[11] There is now compelling evidence for the activation of inflammatory pathways in response to oxidative modification of lipoproteins and alterations to the vascular structure, leading to activation of immune responses.[12]

Early in the atherogenic process, there is increase in adhesion molecule expression within endothelial cells which supports recruitment and attracts binding of leucocytes.[13] Vascular adhesion molecule-1 (VCAM-1) in particular have an important role in recruitment of monocytes to the atheroma, which then differentiates into macrophages, a process which involves internalisation modified lipid particles resulting in foam cell formation.[10] Foam cells contribute to the inflammatory process by secreting pro-inflammatory cytokines, and through the release of growth factors and matrix metalloproteinases (MMP), contributes to atheroma progression and plaque instability.[14, 15]

T lymphocytes are also key contributors to the inflammatory process of atherosclerosis and their recruitment and binding are also enhanced by VCAM-1.[10] Within the atheroma, T lymphocytes differentiate into T helper cells when presented with antigens often generated during oxidative modification of lipoproteins.[16] Pro-

inflammatory T helper cells tend to predominate within the atheroma, producing pro-inflammatory cytokines, some of which can in turn enhance macrophage expression of tissue factors, MMP and pro-inflammatory cytokines.[17]

Nuclear factor κB (NF-κB) is a major transcription factor in inflammatory response, and its activation has been demonstrated in atherosclerotic lesions involving various cell types including smooth muscle cells, macrophages and endothelial cells. NF-κB is involved at different stages along the inflammatory process underlying atherosclerosis, from LDL modification, to production of chemokines, expression of adhesion molecules, and activation of endothelial cells.[18]

In addition to atheroma formation and progression, inflammation also contributes to plaque disruption and rupture. This can occur through activation of MMP as described above leading to degradation of the sub-endothelial membrane and superficial erosion.[19] Furthermore, pro-inflammatory cytokines can inhibit production of collagen which provides the tensile strength of fibrous cap crucial for plaque stability,[20] and adding to this, MMP also have collagenolytic properties and their overexpression have been found in atheromatous plaques,[21] resulting in an atheroma of weak structure susceptible to rupture.

1.2.2 OXIDATIVE STRESS

Oxidative stress is closely linked to inflammation and is central to the atherosclerosis process. It can arise from common risk factors for CVD such as smoking, hypercholesterolaemia, hypertension, type 2 diabetes, and obesity through generation of reactive oxygen species from endothelial cells, vascular smooth muscle and adventitial cells.[22] Oxidative stress reflects the imbalance between oxidant and anti-oxidant mechanisms culminating in generation of reactive oxygen species (ROS) (Figure 1.1). Key components of the oxidant system include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, lipoxygenase, mitochondrial superoxide, nitric oxide synthase, and myeloperoxidase; and once activated, result in generation of ROS such as superoxide anions and hydrogen peroxide.[23] The anti-oxidant system, conversely, reduces the oxidative

process through removal of ROS. Superoxide dismutases convert superoxides to hydrogen peroxide which is then further converted into water and oxygen by catalases.[24] Paraoxonases and nitric oxide (NO) are also a part of the anti-oxidant system and have significant roles in inhibiting lipoprotein oxidation and reducing oxidative stress.[25, 26] ROS exerts a range of atherogenic effects, and of great importance are its interaction with NO and its effect on lipoprotein modification.

The anti-atherogenic effects of NO are well documented. NO is released from the endothelium as a defensive mechanism against atherogenic vascular injury.[27] It exerts protective effects against oxidative modification of low-density lipoprotein (LDL), immune activation and leucocyte migration, platelet aggregation and adherence, and vascular smooth muscle proliferation.[27, 28] NO is a potent vasodilator, as well as a regulator of vascular tone and function of both large and small arteries.[29, 30] Superoxides interact highly with NO leading to reduction in NO bioavailability and, along with it, its vasoprotective functions.[30] Furthermore, ROS can directly impact on endothelial function, reducing NO production.[31] Along with its effect on MMP activation, there are consequential alterations in vascular structure and extracellular matrix which form the foundation for atherosclerosis.[32]

Interaction between ROS and LDL results in oxidative modification and generation of oxidised LDL (OxLDL)[33] which then triggers a cascade of pathological processes including enhanced expression of cell adhesion molecules and recruitment of leucocytes on endothelial cells, leading to immune activation and inflammation. OxLDL in turn promotes further ROS generation within endothelial cells, completing the vicious circle.[23]

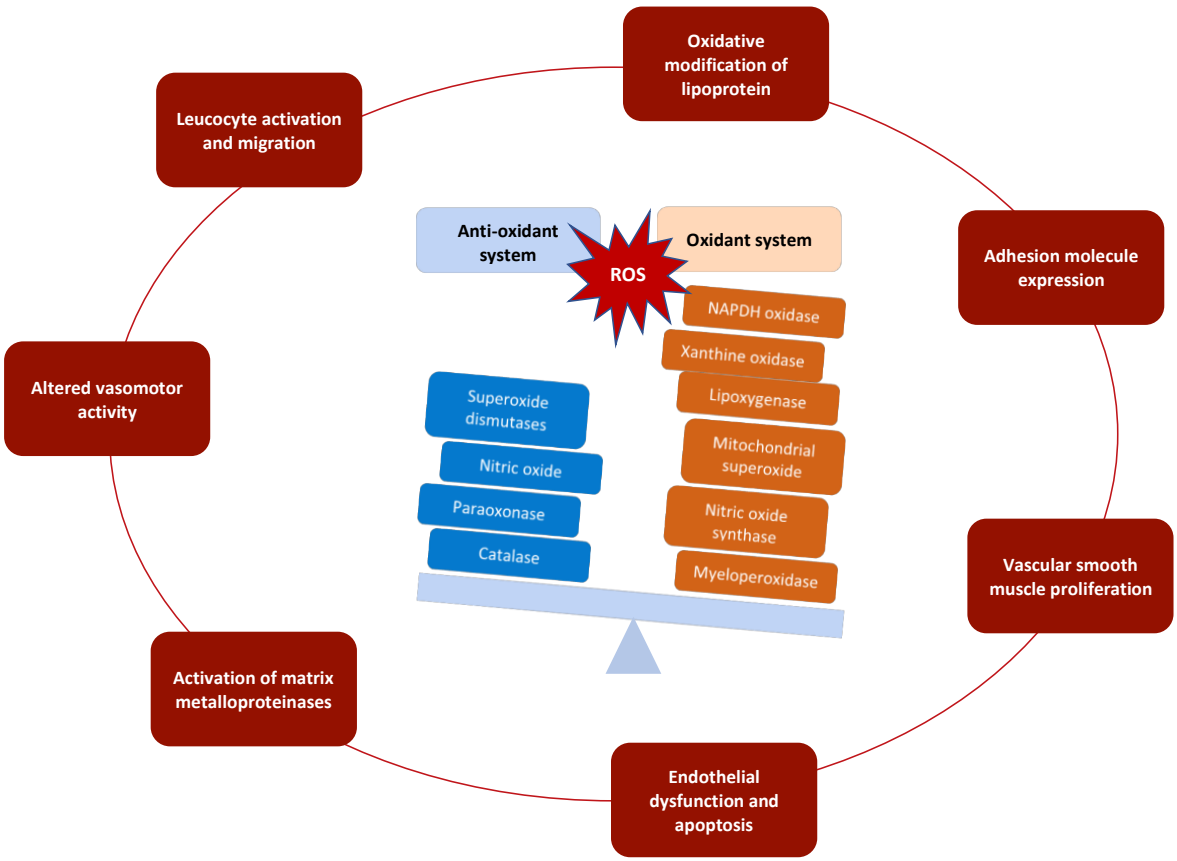


Figure 1.1. Generation of reactive oxygen species in oxidative stress.

The imbalance in anti-oxidant and oxidant systems leading to generation of ROS and cascade of pathophysiological processes underlying atherosclerosis.

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

Data derived from review of information in different articles.[22, 23, 32, 34]

1.2.3 LIPOPROTEIN MODIFICATION

Oxidative modification of lipoproteins, particularly LDL, is crucial in atherogenesis.[35] Early in the atherogenic process, there is accumulation of lipoproteins within the arterial intima which are retained through the interaction between extracellular proteoglycans and apoB-containing lipoproteins.[36] Within the intima, these lipoproteins are exposed to ROS generated from oxidative stress. The LDL particle consists of free cholesterol, cholesteryl esters, phospholipids and triglycerides; of which the acyl chains and sterol of cholesteryl esters and free cholesterol, and

triglycerides are prone to oxidative modification, as are the amino acid residues of the apolipoprotein B-100 (apoB) component.[35] Its molecular composition is therefore one which makes it vulnerable to oxidative modification to form oxidised LDL (OxLDL).

In addition to being internalised at higher rate by macrophages,[37] OxLDL also exerts a wide range of pro-atherogenic effects, which include enhancing expression of adhesion molecule[38] and growth factors,[39] promoting recruitment and proliferation of monocytes and macrophages,[40] increasing secretion of MMP,[41] and promoting vascular cell apoptosis through its cytotoxic properties.[42] Oxidation of LDL leads to generation of a variety of oxidation-specific epitopes (OSE) which are pro-inflammatory and pro-atherogenic such as oxidised phospholipids (OxPL).[43] OxPL is a key contributor to the atherosclerotic process, having major roles in inflammatory cascade activation,[44] plaque destabilisation,[45] and regulation of endothelial cells, smooth muscle cells, macrophages, and platelets within the vascular wall.[46]

Beyond oxidative modification, LDL particles can undergo glycation which can result in enhanced atherogenicity.[47] As glycation of LDL involves epitopes in close proximity to its receptor-binding site,[48] its recognition by the LDL receptor can be altered resulting in reduced clearance from the circulation.[49] Glycated LDL are therefore more likely to be taken up by macrophages and endothelial cells.[50] Furthermore, susceptibility of LDL to oxidative modification may be increased following glycation.[51]

1.2.4 HIGH-DENSITY LIPOPROTEIN

The inverse relationship between high-density lipoprotein cholesterol (HDL-C) and incidence of CVD was established in the Framingham study and subsequent prospective epidemiological studies.[52, 53] Despite this, randomised trials of pharmacological therapies such as niacin[54, 55] and CETP inhibitors[56, 57] have failed to demonstrate CVD outcome benefits with elevation of HDL-C levels. Furthermore, genetic variants associated with low HDL-C levels have not been reliably linked with CVD.[58-60] Likewise, genetic tendencies for higher HDL-C levels did not translate into

lowered CVD risk,[61] suggesting a greater importance of HDL functionality rather than its cholesterol content in influencing CVD risk.

HDL is a lipoprotein particle of complex heterogenous size and structure formed through lipidation of its main protein component apolipoprotein A-I (apoA-I).[62] HDL has a multitude of atheroprotective functions including facilitating reverse cholesterol transport, and exerting anti-inflammatory, anti-oxidation, anti-thrombotic, anti-glycation, and anti-apoptotic effects (Figure 1.2). Reverse cholesterol transport is one aspect of HDL function which has generated great interest and it relates to the removal of cholesterol from peripheral cells which is then transported to the liver for excretion, a process involving passive diffusion and active transport via ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1), scavenger receptor class B type 1 (SR-B1), and cholesteryl ester transfer protein (CETP).[63]

Beyond the capacity for transporting cholesterol, HDL also has anti-inflammatory and anti-oxidant properties. The apoA-I component on HDL is capable for removing lipid hydroperoxides from LDL, preventing it from undergoing oxidative modification.[64, 65] This protective mechanism is also aided by enzymes such as paraoxonase-1 (PON1) on HDL.[66] In addition to protecting against oxidation of LDL, HDL also inhibits activation of the inflammatory pathways triggers by OxLDL through suppression of adhesion molecule expression on endothelial cells and production of monocyte chemotactic protein-1 (MCP-1) thereby reducing migration and infiltration of monocytes and macrophages.[67] However, within a setting of chronic inflammation or during an acute phase response, HDL can become dysfunctional and even exhibit pro-inflammatory properties.[68] This has been demonstrated in patients with type 2 diabetes and metabolic syndrome.[69, 70]

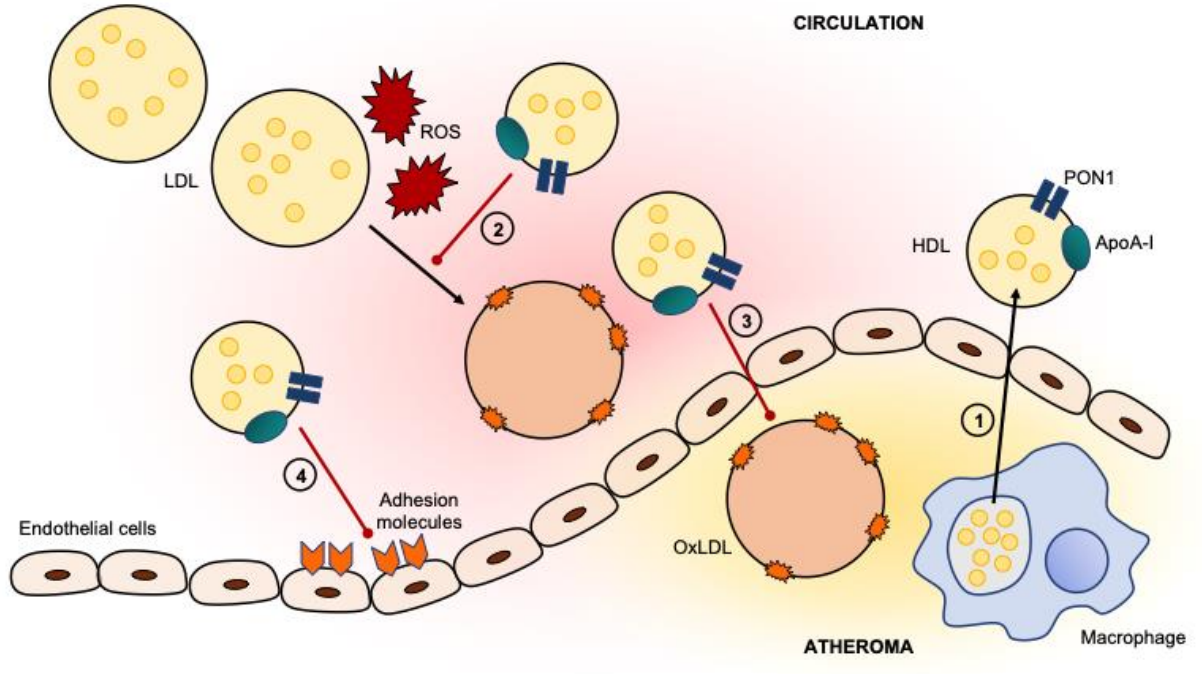


Figure 1.2. Atheroprotective functions of HDL.

Atheroprotective functions of HDL include 1) facilitating reverse cholesterol transport in which transfer of cholesterol from peripheral cells to HDL (cholesterol efflux) is the first step, 2) inhibiting oxidative modification of LDL by ROS, 3) suppression of immune and inflammatory activation by OxLDL, and 4) suppression of adhesion molecule expression on endothelial cells.

Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OxLDL, oxidised LDL; PON1, paraoxonase-1; ROS, reactive oxygen species.

Data derived from review of information in different articles.[63-67]

1.3 ATHEROSCLEROTIC CARDIOVASCULAR DISEASE: RISK MARKERS

The use of cardiovascular risk markers forms a key aspect of cardiovascular medicine, which allows for risk stratification of patients with increasing accuracy to guide treatment strategies. Studies into risk markers also serve to provide further insights into the disease process that form the platform for identifying therapeutic targets. Beyond the classical risk factors of hypertension, dyslipidaemia, diabetes, and smoking, there is great interest in identification of novel biomarkers that could

aid CVD risk prediction and improve understanding of the complex underlying pathophysiology with the aim of developing novel risk modification therapies.

1.3.1 MARKERS OF LDL MODIFICATION

1.3.1.1 OXIDISED LDL

OxLDL has been linked with a wide spectrum of CVD. Multiple studies have demonstrated higher levels of OxLDL among patients with CHD.[71-76] Importantly, significant elevations in OxLDL were observed in the acute phase following acute myocardial infarction,[77-79] supporting its role in plaque progression and rupture. Indeed, in support of this, acute elevations of OxLDL were also seen following percutaneous intervention for coronary occlusion.[80, 81] In a number of studies, OxLDL levels was also able to differentiate between severity of CHD, with markedly higher levels being demonstrated in unstable compared to stable angina which was also found in association with presence of complex plaques on angiography.[82-84] Somewhat conflictingly, similar predictive value of OxLDL levels were not consistently demonstrated in a number of observational studies.[85, 86]

Significant associations have also been demonstrated between elevated OxLDL and carotid artery intima thickness, and likewise, higher levels were also found in patients with carotid atherosclerosis. Similar to CHD, OxLDL levels were found to be significantly higher both in circulation and within plaques of unstable compared to stable lesions.[45, 87] Comparable observations were also made in patients with PVD where strong associations between OxLDL levels and the presence and severity of femoral atherosclerosis.[88] OxLDL was also found to be associated with systolic and diastolic blood pressure in hypertension even in the absence of CHD.[89, 90]

1.3.1.1.1 MEASUREMENT OF OXIDISED LDL

OxLDL can generally be measured using direct and indirect methods. The direct method utilises monoclonal antibodies directed at epitopes on OxLDL while the indirect method detects antibodies generated against OxLDL.

The murine monoclonal antibody 4E6 was the most commonly used and is directed against an epitope which is generated from substitution of lysine residues with aldehydes.[91] Despite being widely used in clinical studies, this assay has its limitations, and most importantly, has been shown that its binding to OxLDL can be competed for by unoxidised LDL at higher concentrations, suggesting that it detects both OxLDL and unoxidised LDL.[92] This is further proven by the increase in OxLDL measured following addition of unoxidised LDL to samples.[93] An alternative method using monoclonal antibody DLH3 which binds to oxidised phosphatidylcholine was also introduced.[94] This assay generates a ratio of oxidised particles on the LDL fraction, and therefore requires isolation of LDL, limiting its wider application.

Indirect measures of OxLDL involves measurement of antibodies and immune complexes (IC) against malondialdehyde-modified LDL (MDA-LDL).[95, 96] IgG antibodies against OSE tend to reflect exposure to antigens generated from oxidation whereas IgM antibodies are natural antibodies that offer protection against activation of inflammatory and immune responses.[97] Associations between IgG MDA-LDL and IgG apoB-IC and cardiovascular events are inconsistent,[98, 99] and importantly, titres of autoantibodies against oxidation-specific epitopes are greatly influenced by age, sex, ethnicity, and hereditary factors.[100]

1.3.1.2 OXIDISED PHOSPHOLIPIDS ON APOB-CONTAINING LIPOPROTEINS AND LIPOPROTEIN(A)

OxPL are generated during the process of LDL oxidation and are key components of OxLDL , apoptotic cells and atherosclerotic lesions.[46] OxPL circulate and bind preferentially to lipoprotein(a) [Lp(a)] as its main lipoprotein carrier.[101, 102] Lp(a) is a complex lipoprotein composed of apolipoprotein(a) [apo(a)] covalently linked with a

single molecule of apoB via a disulphide bond (Figure 1.3).[103] Lp(a) levels are inversely related to the isoform size of its apo(a) component which is highly variable depending on the genetically determined number of kringle IV (KIV) type 2 (KIV-2) repeats.[104] OxPL-apoB are bound to Lp(a) on both the lipid phase of apoB, as well as the apo(a) component.[105] OxPL can be measured on apoB-containing lipoproteins (OxPL-apoB) and given that majority of OxPL on lipoproteins exists on Lp(a), an apoB-containing lipoprotein, OxPL-apoB and Lp(a) are usually closely correlated.[101]

Lp(a) is an independent and causal risk factor for CVD.[106-108] The link between genetic variants associated with high Lp(a) levels and CVD is unequivocal.[109, 110] Specifically, elevated Lp(a) levels are associated with calcific aortic stenosis and its progression, with substantial risk above the levels of approximately 60 mg/dl.[111-114] With the emergence of antisense oligonucleotides, there is increasing focus on Lp(a) as potentially a modifiable CVD risk factor,[115] and the challenges relating to its measurement discussed below.[116]

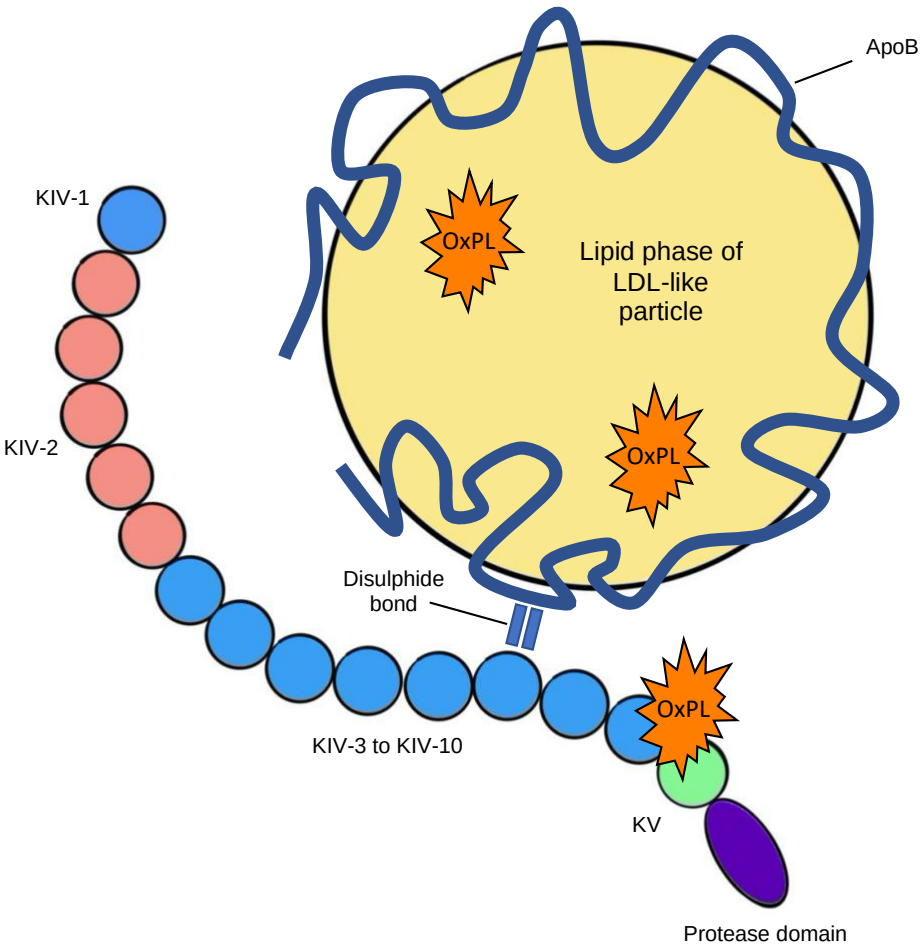


Figure 1.3. Structure of lipoprotein(a) and binding sites for oxidised phospholipids.

The Lp(a) particle consists of an LDL-like particle covalently bound to a single molecule of apo(a) via a disulphide bridge. Lp(a) levels are inversely related to the size of the apo(a) isoform which is dependent on the genetically predetermined number of KIV-2 repeats. Lp(a) is the major lipoprotein carrier of OxPL which are bound to both its lipid phase and the apo(a) component.

Abbreviations: Apo(a), apolipoprotein(a); apoB, apolipoprotein B-100; KIV, kringle IV; kringle V; OxPL, oxidised phospholipids.

The correlation between OxPL-apoB levels and Lp(a) shows a “reverse L” shaped curve following log-transformation, indicating a strong correlation above the Lp(a) threshold of 10 mg/dl but this correlation is lost below this level.[101] Inverse correlations between OxPL-apoB and apo(a) isoform size were also observed which

is independent of ethnicity, and this goes from strongest where the apo(a) isoform size is smallest to progressively weaker in larger isoform sizes.[117] It is therefore postulated that OxPL-apoB levels provide a reflection of the most atherogenic Lp(a) particles. OxPL-apoB is an independent predictor of CVD, and given that it remains an independent predictor when after adjustment for Lp(a) levels in multivariate models particularly in the younger population, is likely a broader reflection of CVD risk beyond merely an indication of Lp(a) levels.[102, 118] High levels of OxPL-apoB have been associated with CHD,[77, 119] cerebrovascular disease,[120, 121] and PVD[120, 122], progression of calcific aortic stenosis,[112, 114, 123] and cardiac-related mortality.[99, 124] OxPL-apoB levels are independently associated with the extent of obstructive coronary artery determined on coronary angiography.[102] Likewise, OxPL-apoB levels predicted progression of both carotid and femoral atherosclerosis in symptomatic patients.[120] Its utility as a predictor of CVD outcomes was assessed in the 10-year prospective epidemiological Bruneck study of 765 patients, and found that OxPL-apoB levels are predictive for CVD events with a hazard ratio of 2.4 (95% CI: 1.3–4.3) in highest compared to lowest tertile of OxPL-apoB levels adjusted for traditional CVD risk factors.[119]

1.3.1.2.1 MEASUREMENT OF LIPOPROTEIN(A)

Measurements of Lp(a) are generally reported in mass as mg/dl or apo(a) particle concentration in nmol/L and standardisation of different methods remain an issue.[125] There are huge variations in the molecular weight and carbohydrate content of the apo(a) component, and similarly the cholesterol and phospholipid content of the apoB component.[116]

The mass assays detect the apo(a) content and reports the total mass of the entire Lp(a) particle by assuming that the apo(a) and lipid components constitute a fixed proportion each, and its accuracy can therefore be greatly affected by variations in contents of each component. Contrastingly, the molar assays measure and report the concentration of apo(a) and therefore Lp(a) particles, overcoming the issue surrounding variations in component mass. Nevertheless, the issue remains that antibodies used to apo(a) in most commercially available assays are polyclonal, and

may detect multiple different sites on a single apo(a) molecule and therefore more likely to overestimate when Lp(a) levels are low with large apo(a) isoform size, and conversely underestimate when Lp(a) levels are high.[116]

These issues have led to the development of an isoform-independent antibody molar method. This assay is currently performed at the Northwest Lipid Metabolism and Diabetes Research Laboratories of the University of Washington and uses a murine monoclonal antibody a-40 which is directed at a single epitope on KIV type 9 (KIV-9).[126] This is the current gold standard for measurement of Lp(a) with values generated being traceable to the WHO/International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference material for Lp(a) and are used to validate commercial assays.[126] This method, however, is not commercially available for widespread utility.

1.3.1.3 GLYCATED LDL

In contrast to OxLDL, the use of glycated LDL in clinical studies assessing cardiovascular risk is limited. Glycation has been proposed as a potentially atherogenic form of modification that can occur in conjunction with oxidation.[47] While there is a lack of evidence for its association with cardiovascular events, higher levels of glycated apoB have been observed in type 2 diabetes and metabolic syndrome.[127]

1.3.2 MARKERS OF HDL FUNCTIONALITY

1.3.2.1 CHOLESTEROL EFFLUX

Cholesterol efflux is the first step in reverse cholesterol transport and refers to the transfer of cholesterol from peripheral cells to HDL.[128] Cholesterol efflux assays measure the capacity of HDL to remove cholesterol from cells, usually cultured from mouse macrophage cell lines.[129] Cholesterol efflux capacity is often used in clinical studies as a measure of HDL function.

Several studies have demonstrated inverse associations between cholesterol efflux capacity and CVD incidence and prevalence independent of traditional CVD risk factors.[130-132] Specifically, it has been negatively associated with both subclinical disease assessed by carotid intima thickness as well as prevalence of clinically significant CHD defined as coronary artery stenosis of over 50% on coronary angiography.[130] In the Dallas Heart Study of 2924 patients with a median follow-up period of 9.4 years, cholesterol efflux capacity was inversely correlated with major cardiovascular events defined as non-fatal myocardial infarction, non-fatal stroke, coronary revascularisation, or CVD death; giving a 67% CVD risk reduction in the highest compared to lowest quartile of cholesterol efflux capacity adjusted for traditional risk factors.[131] More recently, these findings were again corroborated in the EPIC-Norfolk study where comparison was made between 1745 patients and 1749 control participants, with a 20% reduction in risk of incident CVD shown for every standard deviation change in cholesterol efflux capacity, again independent of traditional risk factors and HDL-C levels.[132]

1.3.2.2 PARAOXONASE-1 ACTIVITY

PON1 is synthesised in the liver and is exclusively located on HDL, contributing to the anti-oxidant and anti-inflammatory effects of HDL.[133, 134] PON1 activity is measured most commonly using paraoxon as a substrate.[135] Lower PON1 activity has been observed in patients with CHD,[136] and was inversely correlated with angiographically determined severity and atheroma burden independent of traditional CVD risk factors.[137] More recently, PON1 activity was studied in the PREVEND prospective study of 6902 participants with a mean follow-up of 9.3 years and 730 recorded CVD events.[138] PON1 activity had a log-linear inverse relationship with CVD risk although this was attenuated after adjusting for HDL-C levels.

1.3.2.3 HDL MIRNA

Micro-ribonucleic acids (miRNA) are small non-coding RNAs that negatively regulate messenger RNAs (mRNA) through target transcript degradation or translational inhibition, and are proven to be important regulators of various physiological and metabolic processes.[139] Alterations in miRNA signature have been described in CVD, inflammation, obesity, and related metabolic conditions.[140, 141] More recently, the role of micro-ribonucleic acids (miRNA) as regulators of gene expression influencing HDL function and metabolism has emerged, and HDL have been shown to transport specific miRNAs.[142] HDL-associated miR-233, one of the most abundant miRNA on HDL, in particular has been shown to confer some of the anti-inflammatory effects of HDL where its transfer to endothelial cells led to suppression of adhesion molecule expression.[143]

1.3.3 ANTI-APOA-I AUTOANTIBODY

The presence of autoantibodies against apoA-I (anti-apoA-I IgG) has emerged as an independent predictor of cardiovascular outcome[144, 145] Anti-apoA-I IgG autoantibodies have pro-inflammatory effects through their interaction with immune receptors,[146] and actively contribute to atherothrombosis and plaque vulnerability.[147, 148] Anti-apoA-I IgG levels also have an inverse relationship with PON1 activity in inflammatory disorders,[149] suggesting a potential negative effect on HDL function.

Higher levels of anti-apoA-I IgG autoantibodies have been observed in patients with acute coronary syndrome. It is also associated with higher incidence of recurrent cardiovascular event within the first 12 months. Anti-apoA-I IgG is independently associated with CVD and all-cause mortality in population studies.[145, 150]

1.4 OBESITY

Obesity, defined as a body mass index (BMI) of 30 kg/m^2 or above, is a worldwide public health challenge with a growing prevalence of epidemic proportions, estimated

to affect over 650 million within the adult population in 2016 according to WHO.[151] Excess body fat and weight is closely linked to cardiovascular disease (CVD) and various metabolic conditions of increased CVD risk such as type 2 diabetes, dyslipidaemia, non-alcoholic fatty liver disease (NAFLD), and obstructive sleep apnoea.[152-154] It is also thought that starting from a BMI of around 20 kg/m^2, the increase in cardiovascular co-morbidities and diabetes parallels the increase in BMI.[155] In addition to its negative impact on traditional CVD risk factors such as hypertension, dyslipidaemia, metabolic syndrome, and type 2 diabetes; obesity also has more direct effects on cardiac structure and function.[156]

In a recent large-scale population-based study in the United States, obesity was associated with hazard ratios for lifetime incident CVD of 1.67 (95% CI: 1.55–1.79) and 1.85 (95% CI: 1.72–1.99) for men and women with BMI between 30.0 to 39.9 kg/m^2 respectively, rising to 3.14 (95% CI: 2.48–3.97) and 2.53 (95% CI: 2.20–2.91) for BMI of 40 kg/m^2 and above respectively.[157] Increasing BMI also has a strong association with incident heart failure in the same population. Another population-based study in the Europe, which included patients with no pre-existing CVD, further demonstrated that increasing central adiposity estimated using waist circumference measurements was associated with increasing CVD, with hazard ratios of 1.10 and 1.16 for men and women respectively for every 1 standard deviation increase in waist circumference.[158] Meta-analyses into obesity and mortality have been consistent in demonstrating increase in CVD mortality in patients with BMI of 35 kg/m^2 and above.[159, 160] Although patients in the overweight and mild obesity (BMI 30.0 to 34.9 kg/m^2) categories were associated with lower CVD mortality, this benefit was attenuated after approximately 5 years of follow-up.[160]

Importantly, however, reversible CVD risk factors of hypertension, hypercholesterolaemia, and hyperglycaemia was found to only account for 46% and 76% of the excess risk of raised BMI for CHD and stroke respectively,[161] highlighting the importance of identifying novel CVD risk markers in obesity.

1.4.1 INFLAMMATION AND OXIDATIVE STRESS

The association between inflammation and obesity is well established.[162-164] Inflammation in obesity was first described through the finding of increased adipose tissue pro-inflammatory tumour necrosis factor alpha (TNFα) expression in obese mouse models[165] and subsequently in human obesity[166]. Similar to atherosclerosis but at a systemic level, chronic low-grade inflammation is the hallmark of obesity.[167] Inflammation is the key component in the development of metabolic and cardiovascular complications.[167]

Obesity is driven by a mismatch in energy intake and expenditure, and in order to In order to accommodate the excess energy storage in obesity, adipocytes accumulate triacylglycerols leading to hypertrophy and consequent pathological expansion of white adipose tissue.[168] Adipocyte hypertrophy and consequential remodelling mark the beginning of a cascade of pathophysiological changes including recruitment of macrophages and lymphocytes, and activation of pro-inflammatory cytokines and signalling pathways.[169] The inflammatory process is also driven by production of proinflammatory adipokine leptin from white adipocytes, which further drives cytokine production.[170, 171] Furthermore, low levels of adiponectin, expressed mainly by adipocytes and has established anti-atherogenic properties;[172] are a feature of obesity and are also observed in patients with CVD.[173, 174] The immune system activation and alteration in adipokine expression in adipocytes also result in oxidative stress and generation of ROS.

Closely linked with inflammation, oxidative stress is also driven by immune activation within the adipose tissue, as well as production of adipokines that enhances the oxidant system, leading to generation of reactive oxygen species.[175] Moreover, the anti-oxidant system is also depleted in obesity, specifically superoxide dismutase is found to be lower in obesity compared to normal weight inidividuals.[176] Additionally, ROS and cytokines such as TNFα and interleukin-6 (IL-6) can also suppress adiponectin production,[177, 178] therefore negatively impacting its NO-increasing effects on endothelial cells.[179]

1.4.2 INSULIN RESISTANCE

Insulin resistance is a key component of many of the adverse metabolic effects of obesity. Within the liver and similarly in adipose tissue, metabolic cells (hepatocytes and adipocytes) are closely linked with immune cells (Kupffer cells and macrophages) in an organised architecture allowing for dynamic interactions between both systems.[167] Immune activation and chronic inflammation have a critical role in insulin resistance, and the role of cytokines such as TNFα, which is overexpressed in obesity,[180] in regulating insulin action is well studied.[166, 180, 181] The absence of TNFα has led to improvement in insulin sensitivity in mouse models,[182] and further to this, administration of recombinant TNFα and interleukin-6 (IL-6) in humans has been shown to impair insulin action assessed by whole body glucose uptake.[183] The impact of adipokines on insulin resistance via its effect on inflammation is also well described.[184]

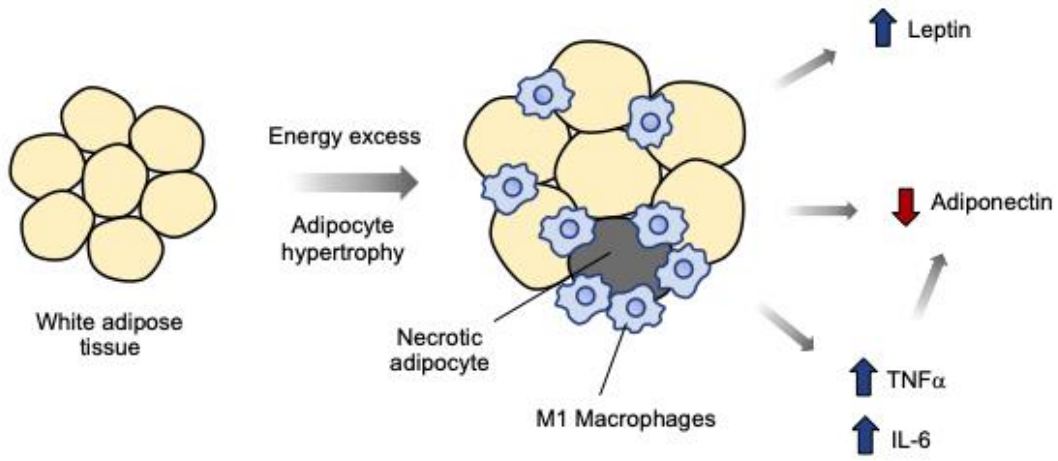


Figure 1.4. Adipocyte hypertrophy.

Mismatch in energy intake and expenditure results in adipocyte hypertrophy, leading to recruitment of pro-inflammatory M1 macrophages, increase in production of cytokines TNFα and IL-6, decrease in adiponectin, and increase in leptin, culminating in insulin resistance. Tissue hypoxia and recruitment of M1 macrophages results in adipocyte necrosis which further drives the immune and inflammatory activation.

Abbreviations: IL-6, interleukin-6; TNFα, tumour necrosis factor alpha

Data derived from review of information in different articles.[169-172]

1.4.3 OBESITY AND ATHEROGENIC MODIFICATION OF LDL

With oxidative stress and inflammation being hallmarks of obesity, the increased risk of atherogenic modification of LDL in obesity is to be expected. Indeed, increased levels of OxLDL are observed in obesity, metabolic syndrome, and diabetes, and are also closely related with fasting glucose, insulin, and insulin resistance across all age groups.[185-187] In support of the role of adipokines in inflammation, oxidative stress, and insulin resistance, OxLDL levels were also negatively correlated with adiponectin.[188]

Increased prevalence of small dense LDL (sdLDL) has also been observed in patients with obesity,[189] metabolic syndrome,[190] and type 2 diabetes.[191] sdLDL circulates longer compared to the larger LDL particles and therefore has longer exposure to atherogenic modification.[192] sdLDL has low antioxidant and lipid composition which is more susceptible to oxidative modification.[193, 194] Moreover, sdLDL is also more susceptible to glycation of apoB, further increasing its atherogenicity.[195]

1.4.4 OBESITY AND LIPOPROTEIN(A)

Despite the association between obesity and CVD risk, of which Lp(a) is an independent causal risk factor for, there is no established associations between obesity and Lp(a) levels. However, Lp(a) levels have been inversely associated with insulin resistance and insulin levels,[196-198] postulated to be a result of insulin-mediated suppression of hepatic apo(a) synthesis.[199] Elevations in Lp(a) have also been observed following dietary-induced weight loss.[200] Similar to Lp(a), the impact of obesity on OxPL-apoB, a likely reflection of the CVD effects of Lp(a), is not established. Given the increase in oxidative stress and higher levels of OxLDL in obesity, one may expect higher levels of OxPL-apoB which require confirmation. The impact of therapies in obesity, especially where reduction in insulin resistance can be

expected, is therefore of great interest given the potential for raising Lp(a) and hence OxPL-apoB, both of which are independent CVD risk factors.

1.4.5 OBESITY AND HDL

Low HDL-C levels are often described in obesity, with an inverse relationship being observed between HDL-C levels and BMI.[201-203] HDL-C levels are also inversely correlated with insulin levels and severity of insulin resistance.[204, 205] Furthermore, levels of apoA-I, its principle protein component, are also frequently low.[206, 207] High triglyceride levels in obesity is a possible mechanism, where CETP facilitates an increase transfer of cholesteryl esters from HDL in exchange for triglycerides, contributing to lower HDL levels.[208] This process also leads to a larger proportion of large triglyceride-rich HDL particles which are more susceptible to hydrolysis by hepatic lipase and subsequent hepatic uptake.[209] Moreover, ApoA-I can also dissociate from triglyceride-rich HDL leading to increased clearance from circulation.[210] Both CETP and hepatic lipase activity are also enhanced in obesity, which would also explain the low HDL-C levels through enhanced clearance.

Beyond HDL-C levels, impairment in HDL can be expected in obesity given the state of chronic inflammation that characterises obesity and the established association between inflammation and dysfunctional HDL impairment. Impairment in reverse cholesterol transport relating to impaired SR-B1 mediated cholesterol efflux and hepatic and adipose tissue cholesterol uptake have been noted in obese mouse models.[211] Similarly, also in mouse models, high levels of TNFα have also been shown to suppress ABCA1 and SR-B1 expression, and also reduce adipocyte cholesterol efflux.[212, 213] In observational human studies, however, there were some inconsistent observations on impaired HDL-mediated cholesterol efflux capacity in obesity, and some of the observed differences in cholesterol efflux may be related to HDL-C levels.[214-216] Lower levels of PON1 activity were also observed in obesity and are independently associated with BMI.[217, 218] Adipokines appear to have an important influence on PON1 activity, with leptin being negatively correlated with PON1 activity and the reverse with adiponectin.[217, 219] PON1 activity is also negatively correlated with markers of metabolic syndrome.[217, 219] In patients with type

2 diabetes, there is also a reduction in PON1 activity, with further reductions noted in the presence of peripheral neuropathy.[135]

The emerging role of HDL-associated miRNA in HDL functionality is one of interest in obesity. Various alterations in miRNA signature in adipose tissues and circulation have been described in obesity and associated metabolic conditions.[220] Among miRNA transported on HDL, increased levels of miR-222 and miR-223 in circulation have been described in obesity,[221, 222] as well as decreased miR-126 in white adipose tissue and isolated fat cells.[223] HDL-associated miR-223 have been shown to decrease following diet-induced weight loss in patients with obesity.[224] The study of HDL-associated miRNA especially in the context of therapies in obesity would help provide newer insights into both the effect of therapy as well as the mechanisms underlying the various functions of HDL.

1.4.6 BARIATRIC SURGERY

Bariatric surgery is associated with favourable effects on CVD risk factors, with significant improvements in blood pressure,[225, 226] dyslipidaemia,[226, 227] type 2 diabetes,[227-229] and obstructive sleep apnoea;[227] and weight reductions sustained in long term studies.[228-230] These improvements in CVD risk factors were translated into CVD outcomes benefits In the Swedish Obese Subjects (SOS) study, where reductions in major adverse cardiovascular events and mortality were observed, with adjusted hazard ratios of 0.47 (95% CI: 0.29–0.76) and 0.67 (95% CI: 0.54–0.83) respectively.[231] A recent nationwide cohort study in the UK using the Clinical Practice Research Datalink database further confirmed improved CVD outcomes with hazard ratios of 0.41 (95% CI: 0.26–0.60) of myocardial infarction, 0.40 (95% CI: 0.18–0.90) for incident heart failure 0.41 (95% CI: 0.27–0.62) and 0.25 (95% CI: 0.18–0.35) for CVD mortality respectively.[232]

Since the first weight reduction surgery performed in 1954,[233] the understanding for its weight and metabolic effects has progressed significantly, and at the same time bariatric procedures have become safer and less-invasive,[234] leading to the increasing preference for its use as the preferred treatment option for severe

obesity.[235] Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy are the two major bariatric procedures described in this thesis (Figure1.5).

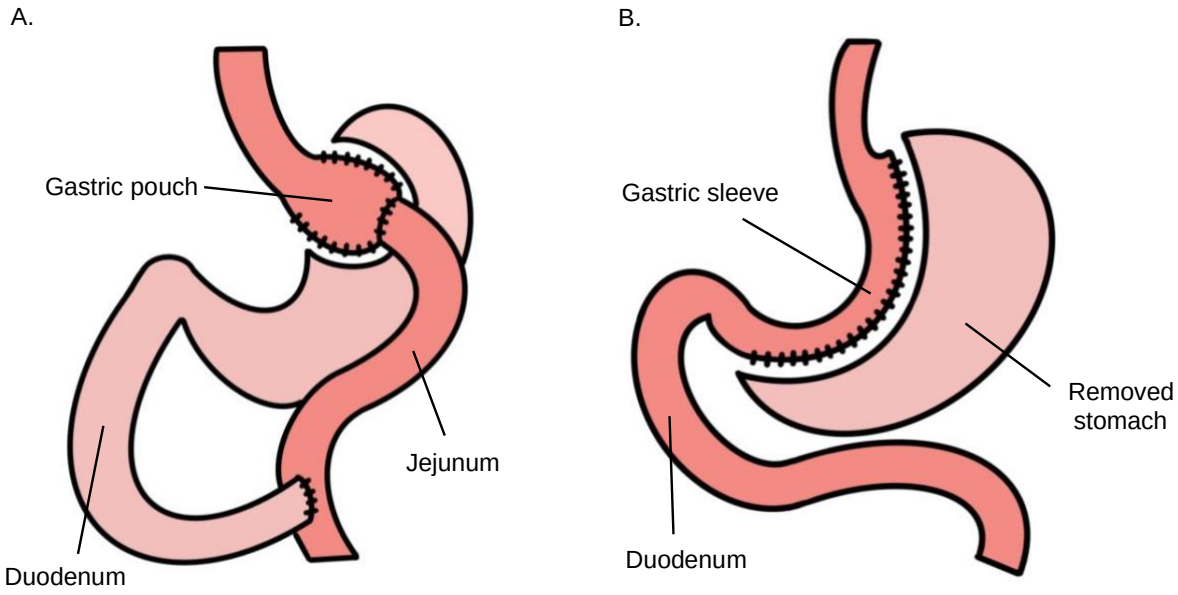


Figure 1.5. Types of common bariatric procedures.

Roux-en-Y gastric bypass (A) is a malabsorptive and restrictive procedure and sleeve gastrectomy (B) is a solely restrictive procedure.

1.4.7 PROPOSED MECHANISMS FOR CARDIOMETABOLIC EFFECTS OF BARIATRIC SURGERY

Reduction in insulin resistance after bariatric surgery was first described in a landmark publication in 1995.[236] Since then multiple studies have unequivocally confirmed sustained glycaemic improvements and indeed remission of type 2 diabetes after bariatric surgery.[227-229] In addition to increased insulin sensitivity, marked improvements in markers of inflammation and oxidative stress have also been widely reported.[237-239] A number of theories have been proposed to explain the metabolic effects of bariatric surgery such as the foregut exclusion hypothesis[240] and hindgut theory.[241]

The foregut exclusion hypothesis proposes an unknown stimulus within the proximal small intestine which provides the trigger for insulin resistance and the bypassing of this segment leads to marked glycaemic improvement.[240] This hypothesis arose from the observation of superior glycaemic effects with duodenojejunal bypass compared to gastrojejunostomy without duodenal bypass in rodents.[240] Furthermore, switching of duodenojejunal bypass to gastrojejunostomy led to deterioration in glycaemic parameters.[240]

The hindgut theory, on the other hand, follows the observation of superior glycaemic control with RYGB and pancreaticobiliary diversion, where there is accelerated delivery of nutrients to the distal ileum, compared to sleeve gastrectomy and other restrictive procedures, suggesting the importance of the distal ileum in regulating insulin sensitivity.[241] The hindgut theory ties in with the role of enterohormones such as incretins. The incretins glucagon-like peptide 1 (GLP-1) and glucose-dependant insulinotropic peptide (GIP) are key contributors to glucose regulation.[242] The incretin effect describes the increase in insulin with decrease in glucagon secretion in response to oral glucose load, and this effect is attenuated in type 2 diabetes, forming the basis for the development of GLP-1 analogues.[242] Increased levels of GLP-1 and GIP are well described following RYGB, suggesting their roles in improving insulin sensitivity.[243, 244]

Further to the foregut exclusion hypothesis and hindgut theory, numerous other mechanisms have been suggested to explain both the metabolic and cardiovascular effects of bariatric surgery. This thesis focuses on improvement in oxidative stress and hepatic steatosis, as well as alterations in miRNA signature after bariatric surgery.

1.4.7.1 HEPATIC STEATOSIS

Obesity is closely associated with the full disease spectrum of non-alcoholic fatty liver disease (NAFLD), often referred to as the hepatic manifestation of metabolic syndrome, ranging from fatty infiltration to steatohepatitis and cirrhosis.[245] Insulin resistance is a key contributor to the progression of NAFLD, along with adipokines

and cytokines.[246] At the same time, lipid accumulation in the liver results in activation of Kupffer cells and macrophages, which in turn further enhances insulin resistance through inflammatory activation and oxidative stress in a vicious circle.[247] Bariatric surgery has been associated with marked reductions in hepatic steatosis, with complete resolution of NAFLD in 66% of patients reported in a meta-analysis of 32 cohort studies including 3093 liver biopsy specimens.[248] Resolution of hepatic steatosis is therefore a very likely mechanism underlying the significant metabolic improvements observe following bariatric surgery. Indeed, in support of this mechanism, resolution of type 2 diabetes and return of first-phase insulin response were observed following very low-calorie diet which resulted in normalisation of hepatic triglyceride content.[249]

1.4.7.2 MIRNA

With the emergence of miRNA as a regulators of physiological and metabolic processes,[139] alterations in miRNA in obesity, related metabolic conditions, and CVD,[140, 141] and the effect of bariatric surgery[250] have garnered increasing interest. Time-dependant alterations in multiple miRNA expression both in circulation and adipose tissues following bariatric surgery have been described in longitudinal studies.[251-253]

This thesis focuses on four miRNA which are associated with obesity, metabolic syndrome and inflammation, and are known to be transported on HDL – miR-24, miR-126, miR-222, and miR-223. Adipose tissue miR-24 level is well correlated with percentage body fat and its expression has been shown to alter in response to hyperglycaemia.[254, 255] MiR-126 is expressed mainly in endothelial cells and is thought to modulate angiogenesis, endothelial cell repair and vascular integrity.[256] Decreased miR-126 expression in white adipose tissue and isolated fat cells has been described in obesity, providing a potential explanation for endothelial dysfunction.[223]

MiR-222 has key involvement in glucose metabolism and is thought to negatively regulate adipose tissue insulin sensitivity.[257] Increased expression of miR-222 in

circulation has been described in obesity,[221, 258] and within the adipose tissue of patients with diabetes and insulin resistance.[257] Its role in glucose regulation is further supported by a study demonstrating its hypoglycaemic effects through induction of pancreatic beta cell proliferation in murine models.[259] Alterations in circulating plasma miR-222 have also been reported following RYGB,[221] although no studies to date have assessed HDL-associated miR-222 following bariatric procedures.

MiR-233 has established roles in immune regulation[260] and increased expression has been described in visceral adipose tissue in obesity.[261] Absence of miR-223 is associated with increased high fat diet induced adipose tissue inflammation and insulin resistance in mouse models.[262] Specifically, transfer of miR-223 from HDL has been shown to directly suppress ICAM-1 expression in endothelial cells, indicating its role in conferring some of the anti-inflammatory effects of HDL. Significant alterations in HDL-associated miR-223 have previously been demonstrated following diet-induced weight loss,[224] but whether similar effects are observed following bariatric surgery are yet to be established.

1.5 POLYCYSTIC OVARIAN SYNDROME

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder characterised by menstrual dysfunction and hyperandrogenisim.[263, 264] It affects approximately 6-10% of women of reproductive age,[265] rising to 20% depending on the diagnostic criteria used.[266] The diagnosis of PCOS can be made based on one of the three sets of commonly used criteria – the National Institute of Health (NIH) criteria,[263] the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) Rotterdam consensus,[267] and the Androgen Excess Society (AES) criteria[268] (Table 1.1).

Table 1.1. Diagnostic criteria for polycystic ovarian syndrome.

NIH criteria*	**ESHE/ASRM**	**AES criteria****
1990	**Rotterdam Consensus**** **2004**	**2009**
Oligomenorrhoea or amenorrhoea	Oligomenorrhoea or anovulation	Oligomenorrhoea or anovulation
Clinical and/or biochemical hyperandrogenism	Clinical and/or biochemical hyperandrogenism	Clinical and/or biochemical hyperandrogenism
	Polycystic ovaries on ultrasound	Polycystic ovaries on ultrasound
Exclusion of other disorders of androgen excess: non-classical congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumours, hyperprolactinaemia, thyroid disorders, drug-induced hyperandrogenism.		

* Both criteria required

* 2 out of 3 criteria required

* Hyperandrogenism and one other criterion required

Table collated using information from published diagnostic criteria.[263, 267, 268]

PCOS is associated with increased CVD risk, owing in part to the increased prevalence of traditional risk factors such as obesity, impaired glucose tolerance, dyslipidaemia, and hypertension.[269, 270] Insulin resistance is a key feature of PCOS despite not being part of any of the three diagnostic criteria, and is independent of obesity.[271] The long-term incidence of metabolic syndrome and diabetes is therefore increased,[272] and being a disease of reproductive age, the lifelong cumulative risk may be substantial. The high prevalence of traditional CVD risk factors, however, has not consistently translated into increased CVD events. A nationwide registry study of Danish women found increased risk of incident CVD in PCOS with a hazard

ratio of 1.6 (95% CI: 1.4–1.8),[273] and likewise, another population-based study of in Australia found hazard ratios of 2.89 (95% CI: 1.68–4.97) for CHD and 2.58 (95% CI: 1.43–4.67) for cerebrovascular disease respectively.[274] Contrastingly, PCOS was not significantly associated with myocardial infarction in meta-analysis of five cohort studies and five case-control studies,[275] and although a further meta-analysis of eight studies undertaken in 2017 (including 128 977 women) did find an increased risk of stroke but this was attenuated following adjustment for BMI.[276] Differences in age and follow-up duration have been suggested as potential explanations for discrepancies in results between studies.[277] Furthermore, PCOS consists of a wide phenotypic spectrum with significant variations in weight, adiposity, severity of insulin resistance and hyperandrogenism, all of which can contribute to CVD risk to differing extent which require further study.

Overall, PCOS provides the opportunity for investigating the impact of factors such as weight and insulin resistance on novel CVD risk markers in the early phase of cardiometabolic disease in a young population.

1.5.1 ALTERATIONS IN LIPID PROFILE

Elevated triglyceride and very low-density lipoprotein cholesterol (VLDL-C), and low HDL-C and apoA-I, which are characteristics of insulin resistance, are often observed in PCOS.[278] There is also a shift towards sdLDL which again is similar to that described in obesity and metabolic syndrome.[279] Higher levels of OxLDL have also been reported,[280] and in a recent study, this was found to be independent of BMI and accompanied by endothelial dysfunction.[281] There are currently no studies into glycation of LDL in PCOS, although with the higher proportion of sdLDL and presence in dysglycaemia, an association with increased glycated LDL could be speculated. Similarly, given the inverse relationship between Lp(a) and insulin resistance, Lp(a) level and its relevance to CVD risk in PCOS is of interest. Overall, the lipoprotein profile in PCOS looks to resemble that of obesity and metabolic syndrome, with insulin resistance being the common feature.

1.5.2 PCOS AND HDL FUNCTIONALITY

There is some evidence for impairment of HDL functionality in PCOS. Decreased cholesterol efflux capacity has been previously described in PCOS in addition to lower apoA-I levels although comparison was made with controls of significantly lower BMI.[282] Another study found improvement in cholesterol efflux capacity following use of combined oral contraceptive pill but not weight loss through intensive lifestyle intervention and cholesterol efflux capacity was correlated inversely with total testosterone.[283] Similarly, decreased PON1 activity has also been reported in PCOS,[284] adding to the evidence of impaired HDL function in PCOS.

1.6 NIACIN

Niacin is also known as nicotinic acid or vitamin B_3. Its utility was limited by the common adverse effect of flushing which was improved with the introduction of extended-release preparation (ERN) and also the addition of laropiprant (LRP).[285] Niacin has a broad range of lipid-modifying effects,[286] of which its ability to raise HDL-C levels had triggered much excitement. However, despite its HDL-C raising effects, ERN has failed to demonstrate CVD outcome benefits in the AIM-HIGH and HPS2 THRIVE trials.[54, 55] In a further meta-analysis of 17 clinical trials which included 35 760 patients, there was an association between niacin monotherapy and reduction of CVD events, with relative risks of 0.74 (95% CI: 0.58–0.96) for acute coronary syndrome, 0.74 (95% CI: 0.59–0.94) for stroke, and 0.51 (95% CI: 0.37–0.72) for coronary revascularisation, suggesting that it retains some benefit in selected population such as those with significant statin intolerance.[287]

In addition to decreases in LDL-C and VLDL-C, niacin had previously been associated with decreases in OxLDL and markers of vascular inflammation such as VCAM-1, MCP-1, and TNFα.[288] ERN and LRP (ERN/LRP) in combination with statin therapy have also been shown to increase cholesterol efflux capacity in addition to HDL-C levels, along with decreases in OxLDL and LpPLA2,[289] suggesting that the beneficial effects of niacin go beyond HDL-C and LDL-C levels.

The lipid-modifying effects of niacin also include an approximate 20% reduction in OxPL-apoB and Lp(a) when used as monotherapy.[290] Although the reduction in OxPL-apoB would be in keeping with reductions in OxLDL and markers of inflammation and oxidative stress in other studies, what is also of interest is its impact when used in combination with other lipid-lowering therapies such as statin where intensity-dependant increases in OxPL-apoB along with Lp(a), but reductions in OxLDL are well-documented.[291-293] Given that both OxPL-apoB and Lp(a) are independent predictors of cardiovascular risk, the impact of different lipid-modifying therapies individually and in combination, and the potential impact on CVD risk are of interest and importance.

1.7 REFERENCES

1. World Health Organization. Cardiovascular diseases2017.

2. Bhatnagar P, Wickramasinghe K, Wilkins E, Townsend N. Trends in the epidemiology of cardiovascular disease in the UK. *Heart.* 2016;102:1945-1952.

3. Bhatnagar P, Wilkins E, Wickramasinghe K, Rayner M, Townsend N. Cardiovascular disease statistics: British Heart Foundation; 2015.

4. Public Health England. Health matters: preventing cardiovacular disease2019.

5. Townsend N, Nichols M, Scarborough P, Rayner M. Cardiovascular disease in Europe 2015: epidemiological update. *Eur Heart J.* 2015;36:2673-2674.

6. Townsend N, Wilson L, Bhatnagar P, Wickramasinghe K, Rayner M, Nichols M. Cardiovascular disease in Europe: epidemiological update 2016. *Eur Heart J.* 2016;37:3232-3245.

7. Virani SS, Alonso A, Benjamin EJ, et al. Heart Disease and Stroke Statistics-2020 Update: A Report From the American Heart Association. *Circulation.* 2020;141:e139-e596.

8. Libby P, Ridker PM, Hansson GK, Leducq Transatlantic Network on A. Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol.* 2009;54:2129-2138.

9. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation.* 2005;111:3481-3488.

10. Libby P. Inflammation in atherosclerosis. *Nature.* 2002;420:868-874.

11. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation.* 2002;105:1135-1143.

12. Taleb S. Inflammation in atherosclerosis. *Arch Cardiovasc Dis.* 2016;109:708-715.

13. Li H, Cybulsky MI, Gimbrone MA, Jr., Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler Thromb.* 1993;13:197-204.

14. Newby AC. Metalloproteinase production from macrophages - a perfect storm leading to atherosclerotic plaque rupture and myocardial infarction. *Exp Physiol.* 2016;101:1327-1337.

15. Galis ZS, Sukhova GK, Kranzhofer R, Clark S, Libby P. Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. *Proc Natl Acad Sci U S A.* 1995;92:402-406.

16. Back M, Yurdagul A, Jr., Tabas I, Oorni K, Kovanen PT. Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities. *Nat Rev Cardiol.* 2019;16:389-406.

17. Lutgens E, Gorelik L, Daemen MJ, et al. Requirement for CD154 in the progression of atherosclerosis. *Nat Med.* 1999;5:1313-1316.

18. de Winther MP, Kanters E, Kraal G, Hofker MH. Nuclear factor kappaB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol.* 2005;25:904-914.

19. Rajavashisth TB, Xu XP, Jovinge S, et al. Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators. *Circulation.* 1999;99:3103-3109.

20. Lee RT, Libby P. The unstable atheroma. *Arterioscler Thromb Vasc Biol.* 1997;17:1859-1867.

21. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest.* 1994;94:2493-2503.

22. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol.* 2003;91:7A-11A.

23. Kattoor AJ, Pothineni NVK, Palagiri D, Mehta JL. Oxidative Stress in Atherosclerosis. *Curr Atheroscler Rep.* 2017;19:42.

24. Yang H, Roberts LJ, Shi MJ, et al. Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apolipoprotein E. *Circ Res.* 2004;95:1075-1081.

25. Tward A, Xia YR, Wang XP, et al. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation.* 2002;106:484-490.

26. Kuhlencordt PJ, Gyurko R, Han F, et al. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation.* 2001;104:448-454.

27. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation.* 2004;109:III27-32.

28. Rubbo H, Trostchansky A, Botti H, Batthyany C. Interactions of nitric oxide and peroxynitrite with low-density lipoprotein. *Biological chemistry.* 2002;383:547-552.

29. Greenstein AS, Khavandi K, Withers SB, et al. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation.* 2009;119:1661-1670.

30. Ganz P, Vita JA. Testing endothelial vasomotor function: nitric oxide, a multipotent molecule. *Circulation.* 2003;108:2049-2053.

31. Chandra S, Romero MJ, Shatanawi A, Alkilany AM, Caldwell RB, Caldwell RW. Oxidative species increase arginase activity in endothelial cells through the RhoA/Rho kinase pathway. *Br J Pharmacol.* 2012;165:506-519.

32. Faraci FM. Oxidative stress: the curse that underlies cerebral vascular dysfunction? *Stroke.* 2005;36:186-188.

33. Ketelhuth DF, Hansson GK. Cellular immunity, low-density lipoprotein and atherosclerosis: break of tolerance in the artery wall. *Thromb Haemost.* 2011;106:779-786.

34. Mueller CF, Laude K, McNally JS, Harrison DG. ATVB in focus: redox mechanisms in blood vessels. *Arterioscler Thromb Vasc Biol.* 2005;25:274-278.

35. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med.* 1989;320:915-924.

36. Boren J, Williams KJ. The central role of arterial retention of cholesterol-rich apolipoprotein-B-containing lipoproteins in the pathogenesis of atherosclerosis: a triumph of simplicity. *Curr Opin Lipidol.* 2016;27:473-483.

37. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A.* 1984;81:3883-3887.

38. Cominacini L, Garbin U, De Santis A, et al. Mechanisms involved in the in vitro modification of low density lipoprotein by human umbilical vein endothelial cells and copper ions. *J Lipid Mediat Cell Signal.* 1996;13:19-33.

39. Stiko-Rahm A, Hultgardh-Nilsson A, Regnstrom J, Hamsten A, Nilsson J. Native and oxidized LDL enhances production of PDGF AA and the surface expression of PDGF receptors in cultured human smooth muscle cells. *Arterioscler Thromb.* 1992;12:1099-1109.

40. Yui S, Sasaki T, Miyazaki A, Horiuchi S, Yamazaki M. Induction of murine macrophage growth by modified LDLs. *Arterioscler Thromb.* 1993;13:331-337.

41. Rajavashisth TB, Liao JK, Galis ZS, et al. Inflammatory cytokines and oxidized low density lipoproteins increase endothelial cell expression of membrane type 1-matrix metalloproteinase. *J Biol Chem.* 1999;274:11924-11929.

42. Sata M, Walsh K. Oxidized LDL activates fas-mediated endothelial cell apoptosis. *J Clin Invest.* 1998;102:1682-1689.

43. Que X, Hung MY, Yeang C, et al. Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice. *Nature.* 2018;558:301-306.

44. Bochkov VN, Oskolkova OV, Birukov KG, Levonen AL, Binder CJ, Stockl J. Generation and biological activities of oxidized phospholipids. *Antioxid Redox Signal.* 2010;12:1009-1059.

45. Nishi K, Itabe H, Uno M, et al. Oxidized LDL in carotid plaques and plasma associates with plaque instability. *Arterioscler Thromb Vasc Biol.* 2002;22:1649-1654.

46. Berliner JA, Leitinger N, Tsimikas S. The role of oxidized phospholipids in atherosclerosis. *J Lipid Res.* 2009;50 Suppl:S207-212.

47. Younis N, Sharma R, Soran H, Charlton-Menys V, Elseweidy M, Durrington PN. Glycation as an atherogenic modification of LDL. *Curr Opin Lipidol.* 2008;19:378-384.

48. Wang X, Bucala R, Milne R. Epitopes close to the apolipoprotein B low density lipoprotein receptor-binding site are modified by advanced glycation end products. *Proc Natl Acad Sci U S A.* 1998;95:7643-7647.

49. Witztum JL, Mahoney EM, Branks MJ, Fisher M, Elam R, Steinberg D. Nonenzymatic glucosylation of low-density lipoprotein alters its biologic activity. *Diabetes.* 1982;31:283-291.

50. Sobal G, Sinzinger H, Menzel EJ. Binding of long-term glycated low density lipoprotein and AGE-albumin by peripheral monocytes and endothelial cells. *J Recept Signal Transduct Res.* 1999;19:267-281.

51. Sobal G, Menzel J, Sinzinger H. Why is glycated LDL more sensitive to oxidation than native LDL? A comparative study. *Prostaglandins Leukot Essent Fatty Acids.* 2000;63:177-186.

52. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med.* 1977;62:707-714.

53. Barter P, Gotto AM, LaRosa JC, et al. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. *N Engl J Med.* 2007;357:1301-1310.

54. Investigators A-H, Boden WE, Probstfield JL, et al. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. *N Engl J Med.* 2011;365:2255-2267.

55. Group HTC, Landray MJ, Haynes R, et al. Effects of extended-release niacin with laropiprant in high-risk patients. *N Engl J Med.* 2014;371:203-212.

56. Schwartz GG, Olsson AG, Abt M, et al. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *N Engl J Med.* 2012;367:2089-2099.

57. Group HTRC, Bowman L, Hopewell JC, et al. Effects of Anacetrapib in Patients with Atherosclerotic Vascular Disease. *N Engl J Med.* 2017;377:1217-1227.

58. Franceschini G, Sirtori CR, Capurso A, 2nd, Weisgraber KH, Mahley RW. A-IMilano apoprotein. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. *J Clin Invest.* 1980;66:892-900.

59. Frikke-Schmidt R, Nordestgaard BG, Stene MC, et al. Association of loss-of-function mutations in the ABCA1 gene with high-density lipoprotein

cholesterol levels and risk of ischemic heart disease. *JAMA.* 2008;299:2524-2532.

60. Trigatti BL, Hegele RA. Rare Genetic Variants and High-Density Lipoprotein: Marching to a Different Drum. *Arterioscler Thromb Vasc Biol.* 2016;36:e53-55.

61. Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet.* 2012;380:572-580.

62. Martin DD, Budamagunta MS, Ryan RO, Voss JC, Oda MN. Apolipoprotein A-I assumes a "looped belt" conformation on reconstituted high density lipoprotein. *J Biol Chem.* 2006;281:20418-20426.

63. Soran H, Hama S, Yadav R, Durrington PN. HDL functionality. *Curr Opin Lipidol.* 2012;23:353-366.

64. Navab M, Hama SY, Cooke CJ, et al. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J Lipid Res.* 2000;41:1481-1494.

65. Navab M, Hama SY, Anantharamaiah GM, et al. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. *J Lipid Res.* 2000;41:1495-1508.

66. Shih DM, Xia YR, Wang XP, et al. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem.* 2000;275:17527-17535.

67. Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. *Circ Res.* 2004;95:764-772.

68. Van Lenten BJ, Hama SY, de Beer FC, et al. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J Clin Invest.* 1995;96:2758-2767.

69. Morgantini C, Natali A, Boldrini B, et al. Anti-inflammatory and antioxidant properties of HDLs are impaired in type 2 diabetes. *Diabetes.* 2011;60:2617-2623.

70. Denimal D, Monier S, Brindisi MC, et al. Impairment of the Ability of HDL From Patients With Metabolic Syndrome but Without Diabetes Mellitus to Activate eNOS: Correction by S1P Enrichment. *Arterioscler Thromb Vasc Biol.* 2017;37:804-811.

71. Holvoet P, Mertens A, Verhamme P, et al. Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol.* 2001;21:844-848.

72. Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation.* 1998;98:1487-1494.

73. Weinbrenner T, Cladellas M, Isabel Covas M, et al. High oxidative stress in patients with stable coronary heart disease. *Atherosclerosis.* 2003;168:99-106.

74. Suzuki T, Kohno H, Hasegawa A, et al. Diagnostic implications of circulating oxidized low density lipoprotein levels as a biochemical risk marker of coronary artery disease. *Clin Biochem.* 2002;35:347-353.

75. Toshima S, Hasegawa A, Kurabayashi M, et al. Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. *Arterioscler Thromb Vasc Biol.* 2000;20:2243-2247.

76. Huang H, Mai W, Liu D, Hao Y, Tao J, Dong Y. The oxidation ratio of LDL: a predictor for coronary artery disease. *Dis Markers.* 2008;24:341-349.

77. Tsimikas S, Bergmark C, Beyer RW, et al. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J Am Coll Cardiol.* 2003;41:360-370.

78. Ehara S, Naruko T, Shirai N, et al. Small coronary calcium deposits and elevated plasma levels of oxidized low density lipoprotein are characteristic of acute myocardial infarction. *J Atheroscler Thromb.* 2008;15:75-81.

79. Fujii H, Shimizu M, Ino H, et al. Oxidative stress correlates with left ventricular volume after acute myocardial infarction. *Jpn Heart J.* 2002;43:203-209.

80. Fefer P, Tsimikas S, Segev A, et al. The role of oxidized phospholipids, lipoprotein (a) and biomarkers of oxidized lipoproteins in chronically occluded coronary arteries in sudden cardiac death and following successful percutaneous revascularization. *Cardiovasc Revasc Med.* 2012;13:11-19.

81. Itabe H, Obama T, Kato R. The Dynamics of Oxidized LDL during Atherogenesis. *J Lipids.* 2011;2011:418313.

82. Anselmi M, Garbin U, Agostoni P, et al. Plasma levels of oxidized-low-density lipoproteins are higher in patients with unstable angina and correlated with angiographic coronary complex plaques. *Atherosclerosis.* 2006;185:114-120.

83. Ehara S, Ueda M, Naruko T, et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. *Circulation.* 2001;103:1955-1960.

84. Yamashita H, Ehara S, Yoshiyama M, et al. Elevated plasma levels of oxidized low-density lipoprotein relate to the presence of angiographically detected complex and thrombotic coronary artery lesion morphology in patients with unstable angina. *Circ J.* 2007;71:681-687.

85. Rossi GP, Cesari M, De Toni R, et al. Antibodies to oxidized low-density lipoproteins and angiographically assessed coronary artery disease in white patients. *Circulation.* 2003;108:2467-2472.

86. Segev A, Strauss BH, Witztum JL, Lau HK, Tsimikas S. Relationship of a comprehensive panel of plasma oxidized low-density lipoprotein markers to angiographic restenosis in patients undergoing percutaneous coronary intervention for stable angina. *Am Heart J.* 2005;150:1007-1014.

87. Sigala F, Kotsinas A, Savari P, et al. Oxidized LDL in human carotid plaques is related to symptomatic carotid disease and lesion instability. *J Vasc Surg.* 2010;52:704-713.

88. Langlois MR, Rietzschel ER, De Buyzere ML, et al. Femoral plaques confound the association of circulating oxidized low-density lipoprotein with carotid atherosclerosis in a general population aged 35 to 55 years: the Asklepios Study. *Arterioscler Thromb Vasc Biol.* 2008;28:1563-1568.

89. Frostegard J, Wu R, Lemne C, Thulin T, Witztum JL, de Faire U. Circulating oxidized low-density lipoprotein is increased in hypertension. *Clin Sci (Lond).* 2003;105:615-620.

90. Imazu M, Ono K, Tadehara F, et al. Plasma levels of oxidized low density lipoprotein are associated with stable angina pectoris and modalities of acute coronary syndrome. *Int Heart J.* 2008;49:515-524.

91. Itabe H, Ueda M. Measurement of plasma oxidized low-density lipoprotein and its clinical implications. *J Atheroscler Thromb.* 2007;14:1-11.

92. Holvoet P, Donck J, Landeloos M, et al. Correlation between oxidized low density lipoproteins and von Willebrand factor in chronic renal failure. *Thromb Haemost.* 1996;76:663-669.

93. Tsouli SG, Kiortsis DN, Lourida ES, et al. Autoantibody titers against OxLDL are correlated with Achilles tendon thickness in patients with familial hypercholesterolemia. *J Lipid Res.* 2006;47:2208-2214.

94. Itabe H. Oxidized low-density lipoproteins: what is understood and what remains to be clarified. *Biol Pharm Bull.* 2003;26:1-9.

95. Choi K, Lee HS, Chung HK. Production and characterization of monoclonal antibodies to oxidized LDL. *Exp Mol Med.* 1998;30:41-45.

96. Wu R, de Faire U, Lemne C, Witztum JL, Frostegard J. Autoantibodies to OxLDL are decreased in individuals with borderline hypertension. *Hypertension.* 1999;33:53-59.

97. Binder CJ, Papac-Milicevic N, Witztum JL. Innate sensing of oxidation-specific epitopes in health and disease. *Nat Rev Immunol.* 2016;16:485-497.

98. Ravandi A, Boekholdt SM, Mallat Z, et al. Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study. *J Lipid Res.* 2011;52:1829-1836.

99. Tsimikas S, Willeit P, Willeit J, et al. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J Am Coll Cardiol.* 2012;60:2218-2229.

100. Prasad A, Clopton P, Ayers C, et al. Relationship of Autoantibodies to MDA-LDL and ApoB-Immune Complexes to Sex, Ethnicity, Subclinical Atherosclerosis, and Cardiovascular Events. *Arterioscler Thromb Vasc Biol.* 2017;37:1213-1221.

101. Taleb A, Witztum JL, Tsimikas S. Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark Med.* 2011;5:673-694.

102. Tsimikas S, Brilakis ES, Miller ER, et al. Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. *N Engl J Med.* 2005;353:46-57.

103. Kronenberg F, Utermann G. Lipoprotein(a): resurrected by genetics. *J Intern Med.* 2013;273:6-30.

104. Kraft HG, Lingenhel A, Pang RW, et al. Frequency distributions of apolipoprotein(a) kringle IV repeat alleles and their effects on lipoprotein(a) levels in Caucasian, Asian, and African populations: the distribution of null alleles is non-random. *Eur J Hum Genet.* 1996;4:74-87.

105. Leibundgut G, Scipione C, Yin H, et al. Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a). *J Lipid Res.* 2013;54:2815-2830.

106. Emerging Risk Factors C, Erqou S, Kaptoge S, et al. Lipoprotein(a) concentration and the risk of coronary heart disease, stroke, and nonvascular mortality. *JAMA.* 2009;302:412-423.

107. Nordestgaard BG, Langsted A. Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology. *J Lipid Res.* 2016;57:1953-1975.

108. Durrington PN, Schofield JD, Siahmansur T, Soran H. Lipoprotein (a): gene genie. *Curr Opin Lipidol.* 2014;25:289-296.

109. Clarke R, Peden JF, Hopewell JC, et al. Genetic variants associated with Lp(a) lipoprotein level and coronary disease. *N Engl J Med.* 2009;361:2518-2528.

110. Burgess S, Ference BA, Staley JR, et al. Association of LPA Variants With Risk of Coronary Disease and the Implications for Lipoprotein(a)-Lowering Therapies: A Mendelian Randomization Analysis. *JAMA Cardiol.* 2018;3:619-627.

111. Kamstrup PR, Tybjaerg-Hansen A, Nordestgaard BG. Elevated lipoprotein(a) and risk of aortic valve stenosis in the general population. *J Am Coll Cardiol.* 2014;63:470-477.

112. Capoulade R, Chan KL, Yeang C, et al. Oxidized Phospholipids, Lipoprotein(a), and Progression of Calcific Aortic Valve Stenosis. *J Am Coll Cardiol.* 2015;66:1236-1246.

113. Zheng KH, Tsimikas S, Pawade T, et al. Lipoprotein(a) and Oxidized Phospholipids Promote Valve Calcification in Patients With Aortic Stenosis. *J Am Coll Cardiol.* 2019;73:2150-2162.

114. Yeang C, Wilkinson MJ, Tsimikas S. Lipoprotein(a) and oxidized phospholipids in calcific aortic valve stenosis. *Curr Opin Cardiol.* 2016;31:440-450.

115. Viney NJ, van Capelleveen JC, Geary RS, et al. Antisense oligonucleotides targeting apolipoprotein(a) in people with raised lipoprotein(a): two randomised, double-blind, placebo-controlled, dose-ranging trials. *Lancet.* 2016;388:2239-2253.

116. Tsimikas S, Fazio S, Viney NJ, Xia S, Witztum JL, Marcovina SM. Relationship of lipoprotein(a) molar concentrations and mass according to lipoprotein(a) thresholds and apolipoprotein(a) isoform size. *J Clin Lipidol.* 2018;12:1313-1323.

117. Tsimikas S, Clopton P, Brilakis ES, et al. Relationship of oxidized phospholipids on apolipoprotein B-100 particles to race/ethnicity, apolipoprotein(a) isoform size, and cardiovascular risk factors: results from the Dallas Heart Study. *Circulation.* 2009;119:1711-1719.

118. Tsimikas S, Duff GW, Berger PB, et al. Pro-inflammatory interleukin-1 genotypes potentiate the risk of coronary artery disease and cardiovascular events mediated by oxidized phospholipids and lipoprotein(a). *J Am Coll Cardiol.* 2014;63:1724-1734.

119. Kiechl S, Willeit J, Mayr M, et al. Oxidized phospholipids, lipoprotein(a), lipoprotein-associated phospholipase A2 activity, and 10-year cardiovascular outcomes: prospective results from the Bruneck study. *Arterioscler Thromb Vasc Biol.* 2007;27:1788-1795.

120. Tsimikas S, Kiechl S, Willeit J, et al. Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. *J Am Coll Cardiol.* 2006;47:2219-2228.

121. Byun YS, Yang X, Bao W, et al. Oxidized Phospholipids on Apolipoprotein B-100 and Recurrent Ischemic Events Following Stroke or Transient Ischemic Attack. *J Am Coll Cardiol.* 2017;69:147-158.

122. Bertoia ML, Pai JK, Lee JH, et al. Oxidation-specific biomarkers and risk of peripheral artery disease. *J Am Coll Cardiol.* 2013;61:2169-2179.

123. Capoulade R, Chan KL, Mathieu P, et al. Autoantibodies and immune complexes to oxidation-specific epitopes and progression of aortic stenosis: Results from the ASTRONOMER trial. *Atherosclerosis.* 2017;260:1-7.

124. Tsimikas S, Mallat Z, Talmud PJ, et al. Oxidation-specific biomarkers, lipoprotein(a), and risk of fatal and nonfatal coronary events. *J Am Coll Cardiol.* 2010;56:946-955.

125. Cegla J, Neely RDG, France M, et al. HEART UK consensus statement on Lipoprotein(a): A call to action. *Atherosclerosis.* 2019;291:62-70.

126. Marcovina SM, Albers JJ. Lipoprotein (a) measurements for clinical application. *J Lipid Res.* 2016;57:526-537.

127. Younis NN, Soran H, Sharma R, et al. Small-dense LDL and LDL glycation in metabolic syndrome and in statin-treated and non-statin-treated type 2 diabetes. *Diab Vasc Dis Res.* 2010;7:289-295.

128. Heinecke JW. The not-so-simple HDL story: A new era for quantifying HDL and cardiovascular risk? *Nat Med.* 2012;18:1346-1347.

129. Rohatgi A. High-Density Lipoprotein Function Measurement in Human Studies: Focus on Cholesterol Efflux Capacity. *Prog Cardiovasc Dis.* 2015;58:32-40.

130. Khera AV, Cuchel M, de la Llera-Moya M, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med.* 2011;364:127-135.

131. Rohatgi A, Khera A, Berry JD, et al. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med.* 2014;371:2383-2393.

132. Saleheen D, Scott R, Javad S, et al. Association of HDL cholesterol efflux capacity with incident coronary heart disease events: a prospective case-control study. *Lancet Diabetes Endocrinol.* 2015;3:507-513.

133. Mackness MI, Durrington PN. HDL, its enzymes and its potential to influence lipid peroxidation. *Atherosclerosis.* 1995;115:243-253.

134. Watson AD, Berliner JA, Hama SY, et al. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J Clin Invest.* 1995;96:2882-2891.

135. Abbott CA, Mackness MI, Kumar S, Boulton AJ, Durrington PN. Serum paraoxonase activity, concentration, and phenotype distribution in diabetes mellitus and its relationship to serum lipids and lipoproteins. *Arterioscler Thromb Vasc Biol.* 1995;15:1812-1818.

136. Mackness B, Davies GK, Turkie W, et al. Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype? *Arterioscler Thromb Vasc Biol.* 2001;21:1451-1457.

137. Graner M, James RW, Kahri J, Nieminen MS, Syvanne M, Taskinen MR. Association of paraoxonase-1 activity and concentration with angiographic severity and extent of coronary artery disease. *J Am Coll Cardiol.* 2006;47:2429-2435.

138. Kunutsor SK, Bakker SJ, James RW, Dullaart RP. Serum paraoxonase-1 activity and risk of incident cardiovascular disease: The PREVEND study and meta-analysis of prospective population studies. *Atherosclerosis.* 2016;245:143-154.

139. Ebert MS, Sharp PA. Roles for microRNAs in conferring robustness to biological processes. *Cell.* 2012;149:515-524.

140. Vickers KC, Remaley AT. MicroRNAs in atherosclerosis and lipoprotein metabolism. *Curr Opin Endocrinol Diabetes Obes.* 2010;17:150-155.

141. Schroen B, Heymans S. Small but smart--microRNAs in the centre of inflammatory processes during cardiovascular diseases, the metabolic syndrome, and ageing. *Cardiovasc Res.* 2012;93:605-613.

142. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol.* 2011;13:423-433.

143. Tabet F, Vickers KC, Cuesta Torres LF, et al. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun.* 2014;5:3292.

144. Vuilleumier N, Rossier MF, Pagano S, et al. Anti-apolipoprotein A-1 IgG as an independent cardiovascular prognostic marker affecting basal heart rate in myocardial infarction. *Eur Heart J.* 2010;31:815-823.

145. Antiochos P, Marques-Vidal P, Virzi J, et al. Anti-Apolipoprotein A-1 IgG Predict All-Cause Mortality and Are Associated with Fc Receptor-Like 3 Polymorphisms. *Front Immunol.* 2017;8:437.

146. Pagano S, Satta N, Werling D, et al. Anti-apolipoprotein A-1 IgG in patients with myocardial infarction promotes inflammation through TLR2/CD14 complex. *J Intern Med.* 2012;272:344-357.

147. Pagano S, Carbone F, Burger F, et al. Anti-apolipoprotein A-1 auto-antibodies as active modulators of atherothrombosis. *Thromb Haemost.* 2016;116:554-564.

148. Montecucco F, Vuilleumier N, Pagano S, et al. Anti-Apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability. *Eur Heart J.* 2011;32:412-421.

149. Batuca JR, Ames PR, Isenberg DA, Alves JD. Antibodies toward high-density lipoprotein components inhibit paraoxonase activity in patients with systemic lupus erythematosus. *Ann N Y Acad Sci.* 2007;1108:137-146.

150. Antiochos P, Marques-Vidal P, Virzi J, et al. Association between anti-apolipoprotein A-1 antibodies and cardiovascular disease in the general population. Results from the CoLaus study. *Thromb Haemost.* 2016;116:764-771.

151. World Health Organization. Obesity and overweight2020.

152. Rexrode KM, Carey VJ, Hennekens CH, et al. Abdominal adiposity and coronary heart disease in women. *JAMA.* 1998;280:1843-1848.

153. Larsson B, Svardsudd K, Welin L, Wilhelmsen L, Bjorntorp P, Tibblin G. Abdominal adipose tissue distribution, obesity, and risk of cardiovascular disease and death: 13 year follow up of participants in the study of men born in 1913. *British medical journal.* 1984;288:1401-1404.

154. Pi-Sunyer FX. The obesity epidemic: pathophysiology and consequences of obesity. *Obesity research.* 2002;10 Suppl 2:97S-104S.

155. Willett WC, Dietz WH, Colditz GA. Guidelines for healthy weight. *N Engl J Med.* 1999;341:427-434.

156. Lavie CJ, Arena R, Alpert MA, Milani RV, Ventura HO. Management of cardiovascular diseases in patients with obesity. *Nat Rev Cardiol.* 2018;15:45-56.

157. Khan SS, Ning H, Wilkins JT, et al. Association of Body Mass Index With Lifetime Risk of Cardiovascular Disease and Compression of Morbidity. *JAMA Cardiol.* 2018;3:280-287.

158. Iliodromiti S, Celis-Morales CA, Lyall DM, et al. The impact of confounding on the associations of different adiposity measures with the incidence of

cardiovascular disease: a cohort study of 296 535 adults of white European descent. *Eur Heart J.* 2018;39:1514-1520.

159. Romero-Corral A, Montori VM, Somers VK, et al. Association of bodyweight with total mortality and with cardiovascular events in coronary artery disease: a systematic review of cohort studies. *Lancet.* 2006;368:666-678.

160. Wang ZJ, Zhou YJ, Galper BZ, Gao F, Yeh RW, Mauri L. Association of body mass index with mortality and cardiovascular events for patients with coronary artery disease: a systematic review and meta-analysis. *Heart.* 2015;101:1631-1638.

161. Global Burden of Metabolic Risk Factors for Chronic Diseases C, Lu Y, Hajifathalian K, et al. Metabolic mediators of the effects of body-mass index, overweight, and obesity on coronary heart disease and stroke: a pooled analysis of 97 prospective cohorts with 1.8 million participants. *Lancet.* 2014;383:970-983.

162. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest.* 2005;115:1111-1119.

163. Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. *The American journal of clinical nutrition.* 2006;83:461S-465S.

164. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. *J Clin Invest.* 2011;121:2111-2117.

165. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science.* 1993;259:87-91.

166. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest.* 1995;95:2409-2415.

167. Hotamisligil GS. Inflammation and metabolic disorders. *Nature.* 2006;444:860-867.

168. Fruhbeck G, Mendez-Gimenez L, Fernandez-Formoso JA, Fernandez S, Rodriguez A. Regulation of adipocyte lipolysis. *Nutrition research reviews.* 2014;27:63-93.

169. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003;112:1796-1808.

170. Loffreda S, Yang SQ, Lin HZ, et al. Leptin regulates proinflammatory immune responses. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 1998;12:57-65.

171. Shen J, Sakaida I, Uchida K, Terai S, Okita K. Leptin enhances TNF-alpha production via p38 and JNK MAPK in LPS-stimulated Kupffer cells. *Life sciences.* 2005;77:1502-1515.

172. Ouchi N, Kihara S, Arita Y, et al. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation.* 2001;103:1057-1063.

173. Arita Y, Kihara S, Ouchi N, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun.* 1999;257:79-83.

174. Hotta K, Funahashi T, Arita Y, et al. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol.* 2000;20:1595-1599.

175. Marseglia L, Manti S, D'Angelo G, et al. Oxidative stress in obesity: a critical component in human diseases. *Int J Mol Sci.* 2014;16:378-400.

176. Ozata M, Mergen M, Oktenli C, et al. Increased oxidative stress and hypozincemia in male obesity. *Clin Biochem.* 2002;35:627-631.

177. Furukawa S, Fujita T, Shimabukuro M, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest.* 2004;114:1752-1761.

178. Fasshauer M, Kralisch S, Klier M, et al. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun.* 2003;301:1045-1050.

179. Ouedraogo R, Gong Y, Berzins B, et al. Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo. *J Clin Invest.* 2007;117:1718-1726.

180. Saghizadeh M, Ong JM, Garvey WT, Henry RR, Kern PA. The expression of TNF alpha by human muscle. Relationship to insulin resistance. *J Clin Invest.* 1996;97:1111-1116.

181. Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest.* 1995;95:2111-2119.

182. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature.* 1997;389:610-614.

183. Krogh-Madsen R, Plomgaard P, Moller K, Mittendorfer B, Pedersen BK. Influence of TNF-alpha and IL-6 infusions on insulin sensitivity and expression of IL-18 in humans. *Am J Physiol Endocrinol Metab.* 2006;291:E108-114.

184. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. *Gastroenterology.* 2007;132:2169-2180.

185. Kelly AS, Jacobs DR, Jr., Sinaiko AR, Moran A, Steffen LM, Steinberger J. Relation of circulating oxidized LDL to obesity and insulin resistance in children. *Pediatr Diabetes.* 2010;11:552-555.

186. Park K, Gross M, Lee DH, et al. Oxidative stress and insulin resistance: the coronary artery risk development in young adults study. *Diabetes Care.* 2009;32:1302-1307.

187. Njajou OT, Kanaya AM, Holvoet P, et al. Association between oxidized LDL, obesity and type 2 diabetes in a population-based cohort, the Health, Aging and Body Composition Study. *Diabetes Metab Res Rev.* 2009;25:733-739.

188. Hoogeveen RC, Ballantyne CM, Bang H, et al. Circulating oxidised low-density lipoprotein and intercellular adhesion molecule-1 and risk of type 2 diabetes mellitus: the Atherosclerosis Risk in Communities Study. *Diabetologia.* 2007;50:36-42.

189. Magkos F, Mohammed BS, Mittendorfer B. Effect of obesity on the plasma lipoprotein subclass profile in normoglycemic and normolipidemic men and women. *Int J Obes (Lond).* 2008;32:1655-1664.

190. Fan J, Liu Y, Yin S, et al. Small dense LDL cholesterol is associated with metabolic syndrome traits independently of obesity and inflammation. *Nutr Metab (Lond).* 2019;16:7.

191. Berneis K, Jeanneret C, Muser J, Felix B, Miserez AR. Low-density lipoprotein size and subclasses are markers of clinically apparent and non-apparent atherosclerosis in type 2 diabetes. *Metabolism.* 2005;54:227-234.

192. Packard C, Caslake M, Shepherd J. The role of small, dense low density lipoprotein (LDL): a new look. *Int J Cardiol.* 2000;74 Suppl 1:S17-22.

193. Ohmura H, Mokuno H, Sawano M, et al. Lipid compositional differences of small, dense low-density lipoprotein particle influence its oxidative susceptibility: possible implication of increased risk of coronary artery disease in subjects with phenotype B. *Metabolism.* 2002;51:1081-1087.

194. Tribble DL, Rizzo M, Chait A, Lewis DM, Blanche PJ, Krauss RM. Enhanced oxidative susceptibility and reduced antioxidant content of metabolic precursors of small, dense low-density lipoproteins. *Am J Med.* 2001;110:103-110.

195. Younis NN, Soran H, Pemberton P, Charlton-Menys V, Elseweidy MM, Durrington PN. Small dense LDL is more susceptible to glycation than more buoyant LDL in Type 2 diabetes. *Clin Sci (Lond).* 2013;124:343-349.

196. Marzano L, Colussi G, Del Torre M, Sechi LA, Catena C. Relationships of plasma lipoprotein(a) levels with insulin resistance in hypertensive patients. *Metabolism.* 2014;63:1439-1446.

197. Rainwater DL, Haffner SM. Insulin and 2-hour glucose levels are inversely related to Lp(a) concentrations controlled for LPA genotype. *Arterioscler Thromb Vasc Biol.* 1998;18:1335-1341.

198. Duell PB, Hagemenas F, Connor WE. The relationship between serum lipoprotein(a) and insulinemia in healthy nondiabetic adult men. *Diabetes Care.* 1994;17:1135-1140.

199. Neele DM, de Wit EC, Princen HM. Insulin suppresses apolipoprotein(a) synthesis by primary cultures of cynomolgus monkey hepatocytes. *Diabetologia.* 1999;42:41-44.

200. Berk KA, Yahya R, Verhoeven AJM, et al. Effect of diet-induced weight loss on lipoprotein(a) levels in obese individuals with and without type 2 diabetes. *Diabetologia.* 2017;60:989-997.

201. Despres JP, Moorjani S, Lupien PJ, Tremblay A, Nadeau A, Bouchard C. Regional distribution of body fat, plasma lipoproteins, and cardiovascular disease. *Arteriosclerosis.* 1990;10:497-511.

202. Glueck CJ, Taylor HL, Jacobs D, Morrison JA, Beaglehole R, Williams OD. Plasma high-density lipoprotein cholesterol: association with measurements

of body mass. The Lipid Research Clinics Program Prevalence Study. *Circulation.* 1980;62:IV-62-69.

203. Lamon-Fava S, Wilson PW, Schaefer EJ. Impact of body mass index on coronary heart disease risk factors in men and women. The Framingham Offspring Study. *Arterioscler Thromb Vasc Biol.* 1996;16:1509-1515.

204. Uusitupa M, Siitonen O, Voutilainen E, et al. Serum lipids and lipoproteins in newly diagnosed non-insulin-dependent (type II) diabetic patients, with special reference to factors influencing HDL-cholesterol and triglyceride levels. *Diabetes Care.* 1986;9:17-22.

205. Laakso M, Sarlund H, Mykkanen L. Insulin resistance is associated with lipid and lipoprotein abnormalities in subjects with varying degrees of glucose tolerance. *Arteriosclerosis.* 1990;10:223-231.

206. Bays HE, Toth PP, Kris-Etherton PM, et al. Obesity, adiposity, and dyslipidemia: a consensus statement from the National Lipid Association. *J Clin Lipidol.* 2013;7:304-383.

207. Garrison RJ, Wilson PW, Castelli WP, Feinleib M, Kannel WB, McNamara PM. Obesity and lipoprotein cholesterol in the Framingham offspring study. *Metabolism.* 1980;29:1053-1060.

208. Tall AR. Plasma lipid transfer proteins. *J Lipid Res.* 1986;27:361-367.

209. Ginsberg HN. Insulin resistance and cardiovascular disease. *J Clin Invest.* 2000;106:453-458.

210. Horowitz BS, Goldberg IJ, Merab J, Vanni TM, Ramakrishnan R, Ginsberg HN. Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol. *J Clin Invest.* 1993;91:1743-1752.

211. Duong M, Uno K, Nankivell V, Bursill C, Nicholls SJ. Induction of obesity impairs reverse cholesterol transport in ob/ob mice. *PLoS One.* 2018;13:e0202102.

212. Zhao SP, Dong SZ. Effect of tumor necrosis factor alpha on cholesterol efflux in adipocytes. *Clin Chim Acta.* 2008;389:67-71.

213. Zhang Y, McGillicuddy FC, Hinkle CC, et al. Adipocyte modulation of high-density lipoprotein cholesterol. *Circulation.* 2010;121:1347-1355.

214. Sasahara T, Nestel P, Fidge N, Sviridov D. Cholesterol transport between cells and high density lipoprotein subfractions from obese and lean subjects. *J Lipid Res.* 1998;39:544-554.

215. Autran D, Guerci B, Paul JL, et al. Basal and postprandial serum-promoted cholesterol efflux in normolipidemic subjects: Importance of fat mass distribution. *Metabolism.* 2001;50:1330-1335.

216. Attia N, Fournier N, Vedie B, et al. Impact of android overweight or obesity and insulin resistance on basal and postprandial SR-BI and ABCA1-mediated serum cholesterol efflux capacities. *Atherosclerosis.* 2010;209:422-429.

217. Bajnok L, Seres I, Varga Z, et al. Relationship of serum resistin level to traits of metabolic syndrome and serum paraoxonase 1 activity in a population with a broad range of body mass index. *Exp Clin Endocrinol Diabetes.* 2008;116:592-599.

218. Ferretti G, Bacchetti T, Moroni C, et al. Paraoxonase activity in high-density lipoproteins: a comparison between healthy and obese females. *J Clin Endocrinol Metab.* 2005;90:1728-1733.

219. Bajnok L, Csongradi E, Seres I, et al. Relationship of adiponectin to serum paraoxonase 1. *Atherosclerosis.* 2008;197:363-367.

220. Landrier JF, Derghal A, Mounien L. MicroRNAs in Obesity and Related Metabolic Disorders. *Cells.* 2019;8.

221. Ortega FJ, Mercader JM, Catalan V, et al. Targeting the circulating microRNA signature of obesity. *Clin Chem.* 2013;59:781-792.

222. Wen D, Qiao P, Wang L. Circulating microRNA-223 as a potential biomarker for obesity. *Obes Res Clin Pract.* 2015;9:398-404.

223. Arner E, Mejhert N, Kulyte A, et al. Adipose tissue microRNAs as regulators of CCL2 production in human obesity. *Diabetes.* 2012;61:1986-1993.

224. Tabet F, Cuesta Torres LF, Ong KL, et al. High-Density Lipoprotein-Associated miR-223 Is Altered after Diet-Induced Weight Loss in Overweight and Obese Males. *PLoS One.* 2016;11:e0151061.

225. Schiavon CA, Bersch-Ferreira AC, Santucci EV, et al. Effects of Bariatric Surgery in Obese Patients With Hypertension: The GATEWAY Randomized Trial (Gastric Bypass to Treat Obese Patients With Steady Hypertension). *Circulation.* 2018;137:1132-1142.

226. Buchwald H, Avidor Y, Braunwald E, et al. Bariatric surgery: a systematic review and meta-analysis. *JAMA.* 2004;292:1724-1737.

227. Gloy VL, Briel M, Bhatt DL, et al. Bariatric surgery versus non-surgical treatment for obesity: a systematic review and meta-analysis of randomised controlled trials. *BMJ.* 2013;347:f5934.

228. Adams TD, Davidson LE, Litwin SE, et al. Weight and Metabolic Outcomes 12 Years after Gastric Bypass. *N Engl J Med.* 2017;377:1143-1155.

229. Sjostrom L. Review of the key results from the Swedish Obese Subjects (SOS) trial - a prospective controlled intervention study of bariatric surgery. *J Intern Med.* 2013;273:219-234.

230. Courcoulas AP, Yanovski SZ, Bonds D, et al. Long-term outcomes of bariatric surgery: a National Institutes of Health symposium. *JAMA Surg.* 2014;149:1323-1329.

231. Sjostrom L, Peltonen M, Jacobson P, et al. Bariatric surgery and long-term cardiovascular events. *JAMA.* 2012;307:56-65.

232. Moussa O, Ardissino M, Heaton T, et al. Effect of bariatric surgery on long-term cardiovascular outcomes: a nationwide nested cohort study. *Eur Heart J.* 2020;41:2660-2667.

233. Kremen AJ, Linner JH, Nelson CH. An experimental evaluation of the nutritional importance of proximal and distal small intestine. *Ann Surg.* 1954;140:439-448.

234. Pomp A. Safety of bariatric surgery. *Lancet Diabetes Endocrinol.* 2014;2:98-100.

235. National Institute for Health and Care Excellence. Surgery for obese adults2020.

236. Pories WJ, Swanson MS, MacDonald KG, et al. Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus. *Ann Surg.* 1995;222:339-350; discussion 350-332.

237. Yadav R, Hama S, Liu Y, et al. Effect of Roux-en-Y Bariatric Surgery on Lipoproteins, Insulin Resistance, and Systemic and Vascular Inflammation in Obesity and Diabetes. *Front Immunol.* 2017;8:1512.

238. Tozzo C, Moreira EAM, de Freitas MB, da Silva AF, Portari GV, Wilhelm Filho D. Effect of RYGB on Oxidative Stress in Adults: a 6-Year Follow-up Study. *Obes Surg.* 2020;30:3301-3308.

239. da Silva VR, Moreira EA, Wilhelm-Filho D, et al. Proinflammatory and oxidative stress markers in patients submitted to Roux-en-Y gastric bypass after 1 year of follow-up. *Eur J Clin Nutr.* 2012;66:891-899.

240. Rubino F. Is type 2 diabetes an operable intestinal disease? A provocative yet reasonable hypothesis. *Diabetes Care.* 2008;31 Suppl 2:S290-296.

241. Cummings DE, Overduin J, Foster-Schubert KE, Carlson MJ. Role of the bypassed proximal intestine in the anti-diabetic effects of bariatric surgery. *Surg Obes Relat Dis.* 2007;3:109-115.

242. Holst JJ, Orskov C. The incretin approach for diabetes treatment: modulation of islet hormone release by GLP-1 agonism. *Diabetes.* 2004;53 Suppl 3:S197-204.

243. Morinigo R, Lacy AM, Casamitjana R, Delgado S, Gomis R, Vidal J. GLP-1 and changes in glucose tolerance following gastric bypass surgery in morbidly obese subjects. *Obes Surg.* 2006;16:1594-1601.

244. Laferrere B, Teixeira J, McGinty J, et al. Effect of weight loss by gastric bypass surgery versus hypocaloric diet on glucose and incretin levels in patients with type 2 diabetes. *J Clin Endocrinol Metab.* 2008;93:2479-2485.

245. Cortez-Pinto H, Camilo ME, Baptista A, De Oliveira AG, De Moura MC. Non-alcoholic fatty liver: another feature of the metabolic syndrome? *Clin Nutr.* 1999;18:353-358.

246. Marchesini G, Brizi M, Morselli-Labate AM, et al. Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med.* 1999;107:450-455.

247. Matsuzawa N, Takamura T, Kurita S, et al. Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. *Hepatology.* 2007;46:1392-1403.

248. Lee Y, Doumouras AG, Yu J, et al. Complete Resolution of Nonalcoholic Fatty Liver Disease After Bariatric Surgery: A Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol.* 2019;17:1040-1060 e1011.

249. Steven S, Hollingsworth KG, Al-Mrabeh A, et al. Very Low-Calorie Diet and 6 Months of Weight Stability in Type 2 Diabetes: Pathophysiological Changes in Responders and Nonresponders. *Diabetes Care.* 2016;39:808-815.

250. Langi G, Szczerbinski L, Kretowski A. Meta-Analysis of Differential miRNA Expression after Bariatric Surgery. *J Clin Med.* 2019;8.

251. Alkandari A, Ashrafian H, Sathyapalan T, et al. Improved physiology and metabolic flux after Roux-en-Y gastric bypass is associated with temporal changes in the circulating microRNAome: a longitudinal study in humans. *BMC Obes.* 2018;5:20.

252. Bae YU, Kim Y, Lee H, et al. Bariatric Surgery Alters microRNA Content of Circulating Exosomes in Patients with Obesity. *Obesity (Silver Spring).* 2019;27:264-271.

253. Ortega FJ, Mercader JM, Moreno-Navarrete JM, et al. Surgery-Induced Weight Loss Is Associated With the Downregulation of Genes Targeted by MicroRNAs in Adipose Tissue. *J Clin Endocrinol Metab.* 2015;100:E1467-1476.

254. Nunez Lopez YO, Garufi G, Pasarica M, Seyhan AA. Elevated and Correlated Expressions of miR-24, miR-30d, miR-146a, and SFRP-4 in Human Abdominal Adipose Tissue Play a Role in Adiposity and Insulin Resistance. *Int J Endocrinol.* 2018;2018:7351902.

255. Xiang Y, Cheng J, Wang D, et al. Hyperglycemia repression of miR-24 coordinately upregulates endothelial cell expression and secretion of von Willebrand factor. *Blood.* 2015;125:3377-3387.

256. Wang S, Aurora AB, Johnson BA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell.* 2008;15:261-271.

257. Deiuliis JA. MicroRNAs as regulators of metabolic disease: pathophysiologic significance and emerging role as biomarkers and therapeutics. *Int J Obes (Lond).* 2016;40:88-101.

258. Ortega FJ, Mercader JM, Moreno-Navarrete JM, et al. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care.* 2014;37:1375-1383.

259. Tsukita S, Yamada T, Takahashi K, et al. MicroRNAs 106b and 222 Improve Hyperglycemia in a Mouse Model of Insulin-Deficient Diabetes via Pancreatic beta-Cell Proliferation. *EBioMedicine.* 2017;15:163-172.

260. Taibi F, Metzinger-Le Meuth V, Massy ZA, Metzinger L. miR-223: An inflammatory oncomiR enters the cardiovascular field. *Biochim Biophys Acta.* 2014;1842:1001-1009.

261. Deiuliis JA, Syed R, Duggineni D, et al. Visceral Adipose MicroRNA 223 Is Upregulated in Human and Murine Obesity and Modulates the Inflammatory Phenotype of Macrophages. *PLoS One.* 2016;11:e0165962.

262. Zhuang G, Meng C, Guo X, et al. A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation. *Circulation.* 2012;125:2892-2903.

263. Dunaif A. *Polycystic ovary syndrome*. Boston

St. Louis, Mo.: Blackwell Scientific Publications ;

Distributors, USA and Canada, Mosby-Year Book; 1992.

264. Rotterdam EA-SPcwg. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod.* 2004;19:41-47.

265. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. *Nat Rev Endocrinol.* 2011;7:219-231.

266. Yildiz BO, Bozdag G, Yapici Z, Esinler I, Yarali H. Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. *Hum Reprod.* 2012;27:3067-3073.

267. Rotterdam EA-SPCWG. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril.* 2004;81:19-25.

268. Azziz R, Carmina E, Dewailly D, et al. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. *Fertil Steril.* 2009;91:456-488.

269. Wild RA, Carmina E, Diamanti-Kandarakis E, et al. Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society. *J Clin Endocrinol Metab.* 2010;95:2038-2049.

270. Moran LJ, Misso ML, Wild RA, Norman RJ. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod Update.* 2010;16:347-363.

271. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. *Diabetes.* 1989;38:1165-1174.

272. Wang ET, Calderon-Margalit R, Cedars MI, et al. Polycystic ovary syndrome and risk for long-term diabetes and dyslipidemia. *Obstet Gynecol.* 2011;117:6-13.

273. Glintborg D, Rubin KH, Nybo M, Abrahamsen B, Andersen M. Cardiovascular disease in a nationwide population of Danish women with polycystic ovary syndrome. *Cardiovasc Diabetol.* 2018;17:37.

274. Hart R, Doherty DA. The potential implications of a PCOS diagnosis on a woman's long-term health using data linkage. *J Clin Endocrinol Metab.* 2015;100:911-919.

275. Zhao L, Zhu Z, Lou H, et al. Polycystic ovary syndrome (PCOS) and the risk of coronary heart disease (CHD): a meta-analysis. *Oncotarget.* 2016;7:33715-33721.

276. Zhou Y, Wang X, Jiang Y, et al. Association between polycystic ovary syndrome and the risk of stroke and all-cause mortality: insights from a meta-analysis. *Gynecol Endocrinol.* 2017;33:904-910.

277. Osibogun O, Ogunmoroti O, Michos ED. Polycystic ovary syndrome and cardiometabolic risk: Opportunities for cardiovascular disease prevention. *Trends Cardiovasc Med.* 2020;30:399-404.

278. Wild RA, Rizzo M, Clifton S, Carmina E. Lipid levels in polycystic ovary syndrome: systematic review and meta-analysis. *Fertil Steril.* 2011;95:1073-1079 e1071-1011.

279. Dejager S, Pichard C, Giral P, et al. Smaller LDL particle size in women with polycystic ovary syndrome compared to controls. *Clin Endocrinol (Oxf).* 2001;54:455-462.

280. Macut D, Panidis D, Glisic B, et al. Lipid and lipoprotein profile in women with polycystic ovary syndrome. *Can J Physiol Pharmacol.* 2008;86:199-204.

281. Oncul M, Albayrak M, Sozer V, et al. Polycystic ovary syndrome and endothelial dysfunction: A potential role for soluble lectin-like oxidized low density lipoprotein receptor-1. *Reprod Biol.* 2020;20:396-401.

282. Roe A, Hillman J, Butts S, et al. Decreased cholesterol efflux capacity and atherogenic lipid profile in young women with PCOS. *J Clin Endocrinol Metab.* 2014;99:E841-847.

283. Dokras A, Playford M, Kris-Etherton PM, et al. Impact of hormonal contraception and weight loss on high-density lipoprotein cholesterol efflux and lipoprotein particles in women with polycystic ovary syndrome. *Clin Endocrinol (Oxf).* 2017;86:739-746.

284. Dursun P, Demirtas E, Bayrak A, Yarali H. Decreased serum paraoxonase 1 (PON1) activity: an additional risk factor for atherosclerotic heart disease in patients with PCOS? *Hum Reprod.* 2006;21:104-108.

285. Kamanna VS, Ganji SH, Kashyap ML. The mechanism and mitigation of niacin-induced flushing. *Int J Clin Pract.* 2009;63:1369-1377.

286. Yadav R, France M, Younis N, et al. Extended-release niacin with laropiprant : a review on efficacy, clinical effectiveness and safety. *Expert Opin Pharmacother.* 2012;13:1345-1362.

287. D'Andrea E, Hey SP, Ramirez CL, Kesselheim AS. Assessment of the Role of Niacin in Managing Cardiovascular Disease Outcomes: A Systematic Review and Meta-analysis. *JAMA Netw Open.* 2019;2:e192224.

288. Ganji SH, Qin S, Zhang L, Kamanna VS, Kashyap ML. Niacin inhibits vascular oxidative stress, redox-sensitive genes, and monocyte adhesion to human aortic endothelial cells. *Atherosclerosis.* 2009;202:68-75.

289. Yadav R, Liu Y, Kwok S, et al. Effect of Extended-Release Niacin on High-Density Lipoprotein (HDL) Functionality, Lipoprotein Metabolism, and Mediators of Vascular Inflammation in Statin-Treated Patients. *J Am Heart Assoc.* 2015;4:e001508.

290. Yeang C, Hung MY, Byun YS, et al. Effect of therapeutic interventions on oxidized phospholipids on apolipoprotein B100 and lipoprotein(a). *J Clin Lipidol.* 2016;10:594-603.

291. Ky B, Burke A, Tsimikas S, et al. The influence of pravastatin and atorvastatin on markers of oxidative stress in hypercholesterolemic humans. *J Am Coll Cardiol.* 2008;51:1653-1662.

292. Choi SH, Chae A, Miller E, et al. Relationship between biomarkers of oxidized low-density lipoprotein, statin therapy, quantitative coronary angiography, and atheroma: volume observations from the REVERSAL (Reversal of

Atherosclerosis with Aggressive Lipid Lowering) study. *J Am Coll Cardiol.* 2008;52:24-32.

293. Ndrepepa G, Braun S, von Beckerath N, et al. Oxidized low density lipoproteins, statin therapy and severity of coronary artery disease. *Clin Chim Acta.* 2005;360:178-186.

CHAPTER 2: HYPOTHESES AND AIMS

Atherogenic modification of LDL and HDL dysfunction are key processes that contribute to the development of atherosclerosis and CVD. Biomarkers of LDL modification and HDL functionality are therefore potential risk markers of CVD that help provide insights into the underlying pathophysiology and the impact of risk modification therapies.

OxPL-apoB and Lp(a) are both independent predictors of CVD and are closely associated with Lp(a) being the main lipoprotein carrier for OxPL. OxPL-apoB is thought to reflect the cardiovascular effects of Lp(a) and generation of OxPL is a direct result of oxidative modification of LDL. Meanwhile, inverse associations between Lp(a) and insulin resistance have been reported in the literature. Lipid-lowering therapies have varying effects on levels of OxPL-apoB and Lp(a), and in addition to its HDL-C elevating effects, niacin have also been shown to reduce both OxPL-apoB and Lp(a) levels.

Inflammation results in impairment of HDL function, with cholesterol efflux capacity being the most commonly studied. More recently, HDL have been shown to transport miRNA, suggesting its role in HDL functionality. Modification of HDL may lead to production of pro-atherogenic antibodies and anti-apoA-I IgG autoantibodies have emerged as a novel risk marker for CVD.

Obesity and the metabolic syndrome are closely associated with CVD risk, with inflammation, oxidative stress and insulin resistance being the common denominator. Bariatric surgery results in improvements in CVD outcomes and marked reductions in markers of inflammation, oxidative stress and insulin resistance. Polycystic ovarian syndrome has many of the features of metabolic syndrome and is also characterised by increased insulin resistance. It is associated with various CVD risk factors, and represents a suitable population for assessing the effect of insulin resistance on CVD risk markers.

2.1 HYPOTHESES

The following hypotheses are tested in this thesis:

1. Bariatric surgery results in reduction in oxidative stress, and therefore reduction in OxPL-apoB and markers of OxLDL.
2. Bariatric surgery leads to improvement in HDL functionality and this translates into changes in expression of HDL-associated miRNA.
3. PCOS is associated with lipoprotein oxidation and glycation, and impaired HDL functionality, which are related to underlying insulin resistance.
4. The effect of extended-released niacin on lowering of OxPL-apoB and Lp(a) is attenuated by the potential elevating effects of statin therapy.

2.2 AIMS

The hypotheses above are addressed through the following aims:

1. To assess the effect of bariatric surgery on OxPL-apoB, markers of OxLDL, and Lp(a)
2. To explore the effect of bariatric on the association between OxPL-apoB and Lp(a) and the relationship with underlying factors such as insulin resistance and hepatic steatosis
3. To explore if the effects OxPL-apoB and Lp(a) differ between surgical procedures and presence or absence of type 2 diabetes
4. To assess the difference in markers of LDL modification and HDL functionality between PCOS and controls and the impact of insulin resistance on these biomarkers
5. To explore the effect of PCOS on anti-apoA-I IgG positivity and the relationship between anti-apoA-I IgG and HDL functionality
6. To assess the effect of extended release niacin on OxPL-apoB and Lp(a) when added to statin therapy
7. To compare Lp(a) measurements using different mass assays in a clinical trial

CHAPTER 3: METHODOLOGY

PREFACE

This chapter provides in further detail the methodology used for results chapters four to seven. As this thesis is presented in journal format, there is overlap of content within this chapter and the methods section within each individual results chapter.

3.1 STUDY DESIGN

The results presented in this thesis are generated from three separate studies. The study design for each of the studies are described here.

Bariatric surgery study

This is a prospective observational study of patients with severe obesity. The results generated from this study are presented in chapters 4 and 5.

Patients were recruited from the Salford Royal NHS Foundation Trust (Salford, UK) tier 4 weight management centre. Patient recruitment was undertaken in the pre-operative clinic and the medical weight management clinic. Study visits were conducted at the NIHR/Wellcome Trust Clinical Research Facility and Cardiovascular Trials Unit at Manchester University NHS Foundation Trust (Manchester, UK). Study assessments were undertaken at baseline, 6 months, and 12 months after bariatric surgery and at baseline and 12 months after medical weight management.

The inclusion criteria covered all patients above the age of 18 years with severe obesity being offered bariatric surgery under the NHS. The BMI criteria is in accordance with the National Institute of Health and Care Excellence (NICE) guidance: BMI of 40 kg/m^2 or more, or BMI between 35 kg/m^2 and 40 kg/m^2 in the presence of other significant weight-related disease.[1] Patients attending the medical weight management clinic were recruited as a comparator group.

Patients with active infection, human immunodeficiency virus infection, recent acute coronary syndrome within 6 months, history of autoimmune disease, active or previous malignancy, haematological disorder, and current or previous immunotherapy were excluded. Patients participating in other interventional research trials were also excluded.

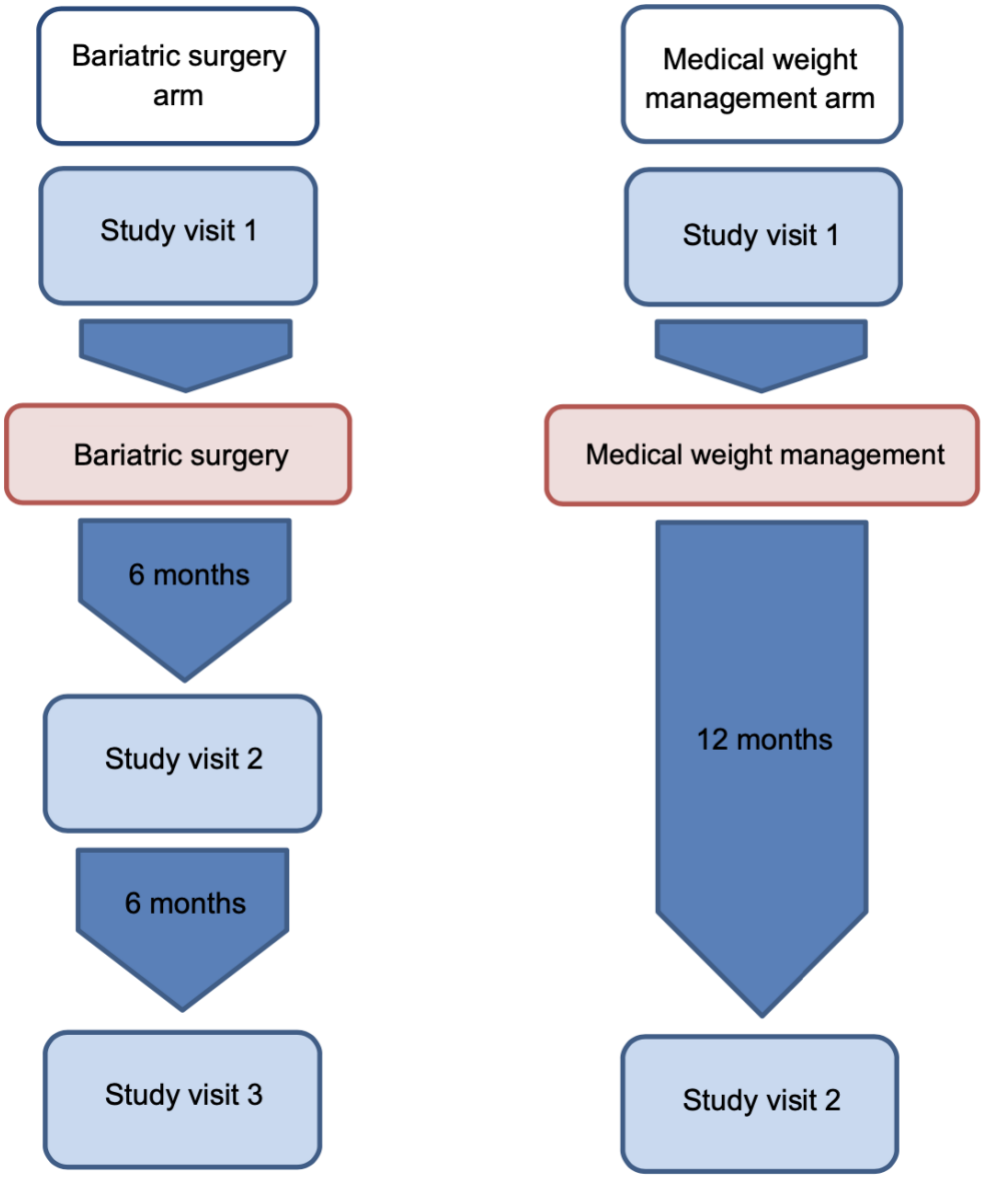


Figure 3.1. Study design for the prospective observational study on patients with severe obesity undergoing bariatric surgery and medical weight management.

Extended-release niacin and laropiprant study

This is a previously completed randomised, double-blind, placebo-controlled crossover trial with trial medication extended-release niacin and laropiprant (ERN/LRP) and image-matched placebo supplied by Merch, Sharp & Dohme Ltd. The primary endpoint of the trial was the increase in HDL-C in statin-treated dyslipidaemic patients following treatment with ERN/LRP compared with placebo. We undertook a post-hoc analysis on the effect of ERN/LRP on Lp(a), OxPL and IgG and IgM autoantibodies and immune complexes to OxLDL. The results generated in this study are presented in chapter 7.

Participants between the age 20 to 75 years on maximal tolerated statin and/or ezetimibe but not achieving a target LDL-C of less than 1.8 mmol/L (70 mg/L) were recruited into the study.

Patients who are pregnant, breast feeding, or with active peptic ulcer disease, renal impairment (defined as estimated glomerular filtration rate less than 60 ml/min/1.73m^2), alanine aminotransferase above 1.5 times upper limit of normal, current use of niacin, fibrates or Omacor, and established allergic reaction to niacin were excluded.

All patients underwent a 4-week placebo run-in period, followed by a 12-week first treatment period where patients were randomised to either ERN/LRP (1g/20mg ERN/LRP for 4 weeks following by an increase to 2g/40mg ERN/LRP for 8 weeks) or placebo. This was then followed by a 4-week placebo washout period and a second treatment period where patients initially randomised to ERN/LRP now received placebo and vice versa. Ezetimibe was discontinued on the first visit while statin dose was unchanged through the study period. Twenty-seven out of the 36 recruited patients completed the study and the patients acted as their own controls.

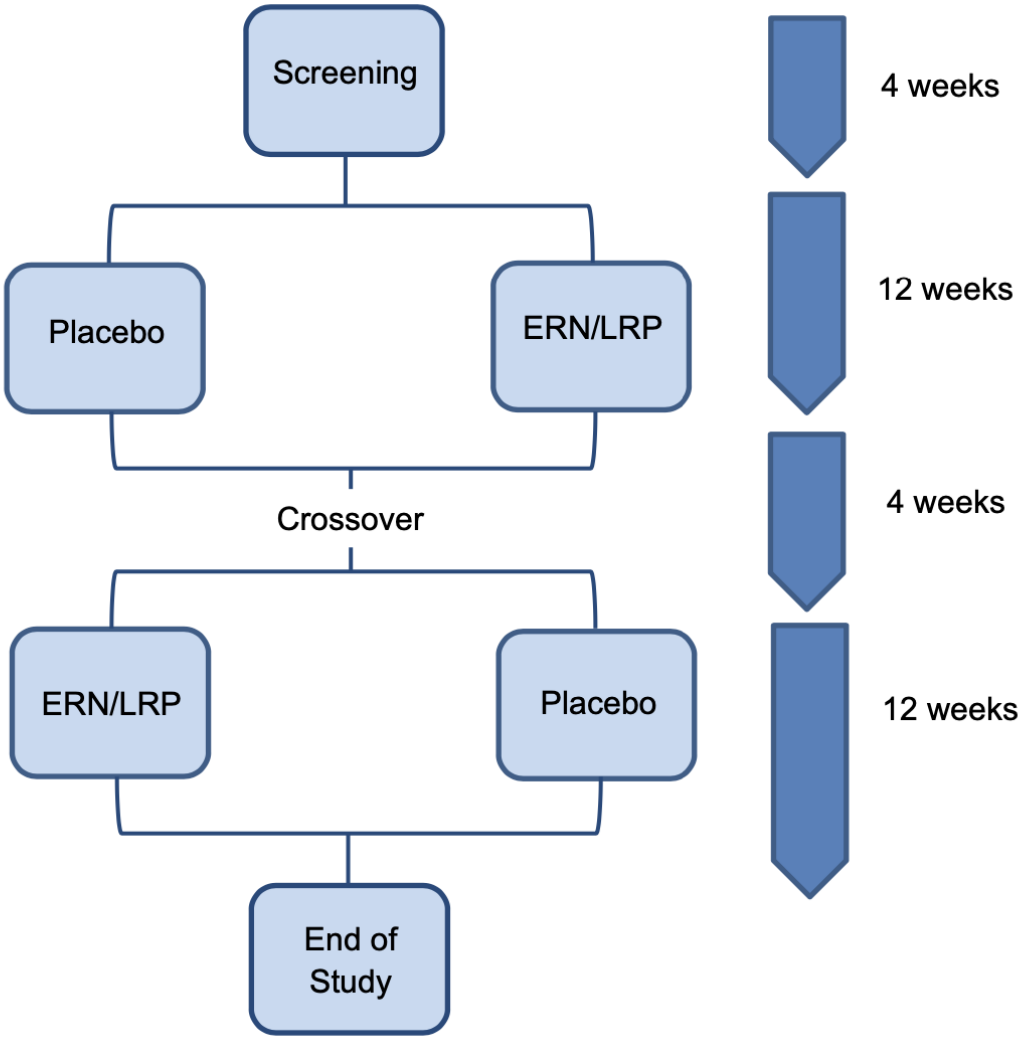


Figure 3.2. Study design for the ERN/LRP randomised, double-blind, placebo-controlled crossover trial.

Polycystic ovarian syndrome study

This is a previously completed cross-sectional study on patients with PCOS and BMI-matched control participants. Consecutive women with PCOS were recruited from the Endocrinology outpatient clinics at Adelaide and Meath Hospital (Tallaght, Dublin, Ireland). The results generated in this study are presented in chapter 6.

The National Institute of Health (NIH) criteria was used to define PCOS which includes chronic oligomenorrhoea (less than nine menstrual cycles per year), and clinical and/or biochemical evidence of hyperandrogenism, following exclusion of other disorders causing the same phenotype.[2] Clinical hyperandrogenism was defined as hirsutism with Ferriman-Gallway score of more than 9, acne, or male pattern alopaecia. Biochemical hyperandrogenism was defined as total testosterone, androstenedione, or dehydroepiandrosterone (DHEAS) above laboratory reference ranges. Thyroid-stimulating hormone, free thyroxine, prolactin, luteinising hormone, follicle-stimulating hormone, oestradiol, and 17-hydroxyprogesterone were measured in all patients to exclude other endocrine disorders. An overnight 1mg dexamethasone suppression test was undertaken for patients with clinical features of cortisol excess.

Control participants were recruited form the general population through advertisements in the study hospital, local schools and community centres. Participants recruited comprises healthy volunteers with normal menstruation, normal testosterone levels, and on no regular medications. Study assessments were carried out in the follicular phase of menstrual cycle.

Exclusion criteria included age less than 18 or more than 40 years, pregnancy, lactation, BMI less than 19 kg/m^2 or more than 50 kg/m^2, recent acute illness, chronic illnesses likely to influence results including diabetes mellitus, medications likely to influence results including hormonal contraception, antihypertensives, lipid-lowering medications, metformin, antiplatelet agents, anti-inflammatory agents, or non-prescription medications.

3.2 ETHICAL APPROVAL

Bariatric surgery study

This study was approved by the Greater Manchester East Research Ethics Committee (REC Reference: 11/NW/0357), the Scientific Advisory Board of the

NIHR/Wellcome Trust Clinical Research Facility, and the Research & Development office of Manchester University NHS Foundation Trust.

Written informed consent was obtained from all study participants prior to entry into the study. Participant information sheets were provided at least 24 hours prior to consent. All aspects of the study were conducted in accordance with the principles of the 1964 Helsinki declaration. Participant information sheets and consent forms are attached in Appendix.

Extended-release niacin and laropiprant study

This study was approved by the Greater Manchester Central Research Ethics Committee and Research, the Scientific Advisory Board of the NIHR/Wellcome Trust Clinical Research Facility, and the Development office of Manchester University NHS Foundation Trust (formerly Central Manchester NHS Foundation Trust) (Study ID: TRED012010, ClinicalTrials.gov Identifier: NCT01054508).

Polycystic ovarian syndrome study

This study has approval from the Research Ethics Committee of the Adelaide and Meath Hospital and St James' Hospital (Dublin, Ireland). Study assessments were undertaken in accordance with the 1964 Helsinki declaration. Written informed consent was obtained from all patients prior to participation in this study. This trial was registered retrospectively at ClinicalTrials.gov (Identifier: NCT001195168).

3.3 CLINICAL ASSESSMENTS

The following clinical assessments were undertaken before, and at 6 months and 12 months after bariatric surgery, and before and at 12 months after medical weight management.

Medical history

A study visit proforma was used to collect information on patient demographics, medical and drug history. In addition to the medical and drug history obtained, a glycosylated haemoglobin (HbA1c) measurement was also undertaken at baseline to identify patients with pre-existing undiagnosed type 2 diabetes (HbA1c $\geq$ 48 mmol/mol) and impaired glucose tolerance (HbA1c 42 to 47 mmol/mol).

Anthropometric measurements

Body mass index was calculated from the height and weight measurements, using the standard equation of weight in kg / (height in m)^2. Waist circumference measurements were taken using a non-distensible flexible tape. Automated blood pressure device was used for assessment of blood pressure, where three measurements were taken and the average of the last two readings calculated.

3.4 BLOOD SAMPLING

For the bariatric surgery and ERN/LRP studies, venous blood samples were obtained from patients between 0800 and 1100 following an overnight fast of at least 12 hours. All laboratory measurements were performed at the lipid research laboratory of the University of Manchester, Core Technology Facility, apart from HbA1c which was measured in the Department of Biochemistry, Manchester University NHS Foundation Trust (Manchester, UK). Apart from HbA1c which was measured on the same of study visit, all laboratory measurements were undertaken at the end of the study.

Separation of serum and plasma

Serum and EDTA-plasma were isolated within 2 hours of collection, using centrifugation (3300 rpm at 4°C at 15 minutes). Aliquots of serum and plasma were

stored at -20 °C or -80°C and each aliquot underwent one freeze-thaw cycle only prior to analysis.

3.5 LIPID PROFILE

Total cholesterol

Measurement of total cholesterol was undertaken using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) method. 3 μl of sample was added to 20 μl of H_2O and 250 μl of reagent. Hydrogen peroxide is released following enzymatic hydrolysis and oxidation by cholesterol esterase, and quinoneimine is generated from 4-aminoantipyrine and phenol in the presence of peroxidase. The cholesterol concentration correlates with the increase in absorbance at 500 nm, which is measured using a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK). The intra- and inter-assay coefficients of variation (CV) were 2.7% and 3.4% respectively.

Triglyceride

Measurement of triglyceride was undertaken using the glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) method. 3 µl of sample was added to 10 µl of H^2O and 290 µl of reagent. Hydrogen peroxide is released following oxidation by glycerol-3-phospate oxidase and quinoeimine in generated from 4-aminoantipyrine and phenol in the presence of peroxidase. The triglyceride concentration correlates with the increase in absorbance at 500 nm, which is measured using a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK). The intra- and inter-assay CV were 3.3% and 3.5% respectively.

High-density lipoprotein cholesterol (HDL-C)

Measurement of HDL-C was undertaken using a second-generation direct homogeneous method. 3 µl of sample was added to 50 µl of 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), and 83 µl of reagent 2 (*N*,*N*-Bis(2-hydroxyethyly)-2-aminoethanesulphonic acid, 4-aminoantipyrine, horse radish peroxidase, sodium azide and surfactants). HDL-cholesterol esters are broken down by polyethylene glycol (PEG)-modified cholesterol esterase into free cholesterol and fatty acids, and cholesterol oxidised by cholesterol oxidase. Hydrogen peroxide is released following oxidation by cholesterol oxidase and reacts with 4-aminoantipyrine and *N*-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline. The concentration of HDL-cholesterol correlates with the increase in absorbance at 600 nm, which is measured using a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK). The intra- and inter-assay CV were 1.2% and 0.9% respectively.

Low-density lipoprotein cholesterol (LDL-C)

LDL-cholesterol was calculated using the Friedewald formula from measurements of total cholesterol, triglyceride, and HDL-cholesterol.[3] This formula is used when serum triglyceride did not exceed 4.5 mmol/l.

LDL-C (mmol/l) = total cholesterol (mmol/l) – HDL-C (mmol/l) – (triglyceride (mmol/l) / 2.19)

Apolipoprotein B-100 (apoB)

Measurement of apoB was undertaken using an immunoturbidimetric immunoassay. 13 µl of sample was added to 30 µl of H_2O, 200 µl of phosphate buffer solution (PBS) polymer solution, 16.7 µl of anti-human apoB antibody, and 53.3 µl of PBS. The concentration of apoB correlates with the increase in absorbance at 340nm,

which is measured using a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK). The intra- and inter-assay CV were 2.2% and 2.6% respectively.

Apolipoprotein A-I (apoA-I)

Measurement of apoA-I was undertaken using an immunoturbidimetric assay. 7 µl of sample was added to 60 µl H_2O, 200 µl of PBS polymer solution, 46.7 µl PBS, and 23.3 µl of purified immunoglobulins from rabbit antiserum (apoA-I from human HDL immunogen). The concentration of apoA-I correlates with the increase in absorbance after at 340 nm, which is measured using a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK).

Proprotein convertase subtilisin/kexin type 9 (PCSK9)

Measurement of PCSK9 was undertaken using a sandwich ELISA method (R&D Systems, Abingdon, UK). Micro-titre plates were pre-coated with capture antibody (lyophilized rat anti-human PCSK9) overnight and 300 µl of reagent diluent and incubated for 60 minutes. Samples and standards were then added and incubated for 2 hours at room temperature followed by 100 µl of detection antibody (lyophilized biotinylated goat anti-human PCSK9 with reagent diluent). After a further 30 minutes of incubation with 100 µl streptavidin-HRP, 100 µl of 3,3’,5,5’-tetramethylbenzidine (TMB) was added to each well at 20 second intervals and incubated for a further 15 to 30 minutes. Stop solution (1 M sulphuric acid) was then added and the OD determined using a microplate reader at 450 nm and the concentration calculated from the standard curve.

Lipoprotein(a) [Lp(a)]

Two different assays were used for the measurement of Lp(a).

The first was a solid phase two-site sandwich ELISA method (Mercodia, Uppsala, Sweden) based on two monoclonal antibodies directed against separate antigenic determinants on the apo(a) molecule. 25 µl of samples and calibrators were added to micro-titre plates pre-coated with anti-apo(a), followed by 50 µl of enzyme conjugate solution. 200 µl of subtrate TMB was then added after 1 hour followed by 50 µl of stop solution. The OD was determined using a microplate reader at 450 nm and the concentration calculated using the standard curve. The concentration of Lp(a) is measured in U/L and converted to mg/dl using a fixed conversion factor of 1 U/L = 0.1254 mg/dl. The intra- and inter-assay CV were 3.3% and 4.0% respectively.

The second was an in-house chemiluminescent ELISA method undertaken at the research laboratory of University of California San Diego.[4, 5] Micro-titre plates were coated with 5 µg/ml of murine monoclonal antibody MB47 to captures all apoB-containing lipoprotein particles. A plasma dilution of 1:400 was added to yield a non-saturating amount of Lp(a). Apo(a) particles linked to apoB were the detected using biotinylated murine monoclonal antibody LPA4. Free apo(a) are not detected on this assay. This assay has a CV of 6.0% to 7.4%.

3.6 MARKERS OF LIPOPROTEIN MODIFICATION

Small dense LDL (sdLDL)

Measurement of sdLDL was undertaken on a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK) using the Randox sLDL-‘Ex-Seiken’ direct test kit. This assay has a CV of less than 3%.

Oxidised low-density lipoprotein (OxLDL)

Measurement of OxLDL was undertaken using a solid phase two-site direct sandwich ELISA method (Mercodia, Uppsala, Sweden) based on two monoclonal antibodies directed against separate antigenic epitopes on oxidised apoB. 25 µl of samples, calibrators, and controls were added to pre-coated micro-titre plates and assay buffer added and incubated on plate shaker for 2 hours at room temperature. 100 µl of enzyme conjugate solution was then added followed by 200 µl of TMB after a further 1 hour of incubation. 50 µl of stop solution was then added after 15 minutes. The OD was determined using a microplate reader at 450 nm and the concentration calculated using the standard curve. The intra- and inter-assay CV were 5.5% and 6.2% respectively.

Oxidised phopsholipids on apolipoprotein B-100 (OxPL-apoB)

Measurement of OxPL-apoB was undertaken using a chemiluminescent immunoassay based on murine monoclonal antibody E06.[6-9] E06 recognises the phosphocholine (PC) group on oxidised but not native phospholipids. A 1:50 dilution of plasma was added to micro-titre plates coated with apoB specific monoclonal antibody MB47. A saturating amount of apoB was added giving equal numbers of apoB particles captured in each well. Biotinylated E06 was then added to determine the content of OxPL-apoB, which reflects the absolute content of OxPL per constant amount of captured apoB, independent of plasma levels of apoB or LDL-C. This assay has a high intra-individual 5-year reproducibility of frozen samples (correlation coefficient 0.78)[10] and pilot-tests showed stable OxPL-apoB levels over 24 hours on ice (intra-class correlation coefficient 0.96)[11] and in frozen samples stored under long term conditions.[12-14]

Oxidised phospholipids on apolipoprotein(a) [OxPL-apo(a)]

OxPL-apo(a) levels were measured in a using a similar method to OxPL-apoB. Instead of monoclonal antibody MB47, LPA4 is used as capture antibody to detect

apo(a).[8] The micro-titre plates were coated with LPA4 antibody, and 1:50 dilution of plasma is added to saturate the plate with apo(a). Biotinylated E06, which does not react with LPA4, is then added to detect the content of OxPL-apo(a), which reflects the absolute content of OxPL per constant amount of captured apo(a).

IgG and IgM to malondialdehyde-modified low-density lipoprotein (MDA-LDL)

A 1:200 dilution of plasma was added to micro-titre plates coated with 5µg/ml of MDA-LDL. Alkaline phosphatase-labeled goat anti-human IgG and IgM (Sigma-Aldrich, St Louis, USA) was then added to detect beinding to MDA-LDL.[15, 16]

IgG and IgM apolipoprotein B-100 immune complexes (apoB-IC)

A 1:50 dilution of plasma was added to micro-titre plates coated with monoclonal antibody MB47, saturating the plate with apoB. Alkaline phosphatase-labeled goat anti-human IgG and IgM (Sigma-Aldrich, St Louis, USA) was then added to detect IgG or IgM autoantibodies bound to the captured apoB (apoB-IC).[15, 16]

Lipoprotein-associated phospholipase A2 (LpPLA2)

Measurement of LpPLA2 was undertaken using a sandwich ELISA method (USCN Lifescience, Buckingham, UK). 100 µl of samples and standards were added to micro-titre plates pre-coated with antibody specific to Lp-PLA2 and incubated for 2 hours at 37°C. 100 µl of working solution containing biotin-conjugated antibody specific to Lp-PLA2 was then added followed by 100 µl working solution containing Avidin conjugated to horseradish peroxidase. 90 µl TMB substrate solution was then added followed by stop solution (1M sulphuric acid) after 15 to 25 minutes. The OD was determined using a microplate reader at 450 nm and the concentration calculated using the standard curve. The intra- and inter-assay CV were 9.5% and 11.5% respectively.

Glycated apoB

Measurement of glycated apoB was undertaken using the Glycacor indirect competitive ELISA (Exocell, Philadelphia, PA, USA) based on mouse monoclonal antibody ES12 which recognises a specific epitope on glycated apoB. Samples and standards were added to Glycacor assay plates pre-coated with standardised preparation of glycated apoB in blocking solution. 50 µl of ES12 anti-glycated apoB was then added and incubated for 1 hour at room temperature, followed by 100 µl of HRP-conjugate. 100 µl colour developer was then added following by 100 µl colour stopper after 10 minutes. The OD was determined using a microplate reader at 450 nm and concentration calculated from the standard curve. The assay has intra- and inter-assay CV of 3.5% and 14.9% respectively.

3.7 MARKERS OF INFLAMMATION

High-sensitivity C-reactive protein (hsCRP)

Measurement of CRP was undertaken using an immunoturbidimetric assay. Sample was added to assay buffer [glycine, sodium chloride, sodium EDTA disodium salt dihydrate and bovine serum albumin (BSA)] and antibody reagent (Latex particles coated with antibody to CRP). The signal generated on turbidimetry is measured at 570 nm using a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK).

Intercellular adhesion molecule 1 (ICAM-1)

Measurement of ICAM-1 was undertaken using a colorimetric sandwich ELISA method (R&D Systems, Abingdon, UK). 100 µl of samples and standards were added to microplate wells pre-coated with capture antibody overnight and incubated for 2 hours at room temperature. 100 µl of detection antibody was then added followed by 100 µl of streptavidin-HRP after a further 2 hours of incubation. 100 µl substrate solution was then added followed by 50 µl stop solution after 20 minutes.

The OD was determined using a microplate reader at 540 nm and the concentration calculated from the standard curve.

3.8 MARKERS OF HDL FUNCTIONALITY

Cholesterol efflux capacity

Cholesterol efflux capacity was measured using a previously validated method.[17] Cell culture of J774A.1 macrophages were prepared in RPMI 1640 medium at 37ºC in a 5% carbon dioxide humidified environment for 3 days. The cells were then collected by centrifugation and suspended in new media (0.4% trypan blue solution) to check for cell count and viability. The cells were plated to a final concentration of 5 x 10^5 cells per ml at 1ml per well. After 24 hours of incubation, the plated cells are then washed and incubated with 0.2 µCi of radiolabelled ^{3}H-cholesterol in RPMI 1640 medium with 0.2% BSA, 100 IU/ml penicillin and 100 g/ml streptomycin in a humidified incubator with 5% carbon dioxide for 24 hours. ABCA1 is upregulated using medium containing 0.3 mM C-AMP (8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium salt) for 4 hours. 2.8% apoB-depleted serum was then added to cells and incubated for 4 hours. The cell media was then collected and dissolved in 0.5 ml of 0.2 NaOH and 2 ml of liquid scintillator added. Radioactivity was determined using liquid scintillation analyser Packard TRI-CARB 2100 TR (Perkin Elmer, Massachusetts, USA).

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

Paraoxonase-1 (PON1) activity

Measurement of PON1 activity was determined using a semi-automated micro-titre plate method on a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK) using paraoxon (*O,O*-diethyl *O*-(4-nitrophenyl)phosphate) (Sigma Chemical, Poole,

UK) as a substrate. 30 µl of sample was added to cryogenic tubes and paraoxone stock solution (36.36 µl paraoxon and 40 ml assay buffer to give 5.5 mmol/l paraoxon) added to the substrate container. Using the PON1 activity measuring programme, a kinetic model is employed to calculate the change in OD at 405 nm per unit time. The intra- and inter-assay CV were 4.9 and 5.3% respectively.

PON1 activity (nmol/ml/min) = OD / min x 2057

Myeloperoxidase (MPO)

Measurement of MPO was undertaken using an in-house antibody sandwich ELISA method based on capture antibody, detection antibody and MPO standards from R&D Systems (Abingdon, UK). 300 µl of reagent diluent (BSA in PBS and *dd* H_20) was added to micro-titre plates pre-coated with capture antibody (lyophilized rat anti-human MPO) overnight and washed with wash buffer (0.05% (v/v) tween-20 in PBS). Samples and MPO standards were then added and incubated for 2 hours at room temperature, followed by 100 µl of detection antibody (lyophilized biotinylated goat anti-human MPO) and incubated for a further 1 hour at room temperature. Streptavidin-HRP (R&D Systems, Abingdon, UK) was then added followed by 100 µl of TMB at 20 second intervals and then 50 µl of stop solution (1 M sulphuric acid). OD was determined using a microplate reader at 630 nm and the concentration calculated from the standard curve.

HDL-associated miRNA

HDL was first isolated from 600 µl of serum by immunoprecipitation using a column containing anti-human apoA-I antibody covalently coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Amersham, UK).[18] The column was then washed with Tris-buffered saline (TBS) to remove proteins non-specifically bound to the beads. HDL was then eluted using stripping buffer (0.1 M acetic acid) and then neutralised with 1 M Tris at pH 11 with a final concentration of

0.11 M. Amicon Ultra-15 centrifuge filter unit and Amicon Ultra-0.5, ultracel-10 membrane were used for further concentration of the samples.

HDL-associated miRNA levels were measured using real-time PCR TaqMan miRNA assays.[18] Qiazol miRNAeasy kits (Qiagen, Hilden, Germany) were used to isolate total RNA from HDL and quantified using spectrophotometry. A TaqMan microRNA reverse transcription kit (Applied Biosystems, California, USA) was used to reverse transcribe purified total RNA. 7.5 µl of the reverse transcription product was used for detecting specific miRNAs using TaqMan miRNA assay kits (Applied Biosystems, California, USA). Values were normalised to both *Caenorhabditis elegans* (Cel) miR-39 (which was spiked into the samples after the Qiazol step) and HDL total protein concentration determined by BCA protein assay kit (Thermo Fisher Scientific, Massachusetts, USA). Results were expressed as $2^{-(Ct(microRNA)-Ct(Cel\text{-}miR\text{-}39))}$.

3.9 MARKERS OF GLYCAEMIA

Glycosylated haemoglobin (HbA1c)

Measurement of HbA1c was undertaken on the same day of study visit at the Department of Biochemistry, Manchester University NHS Foundation Trust (Manchester, UK), using HPLC on a VARIANT II Turbo Haemoglobin Testing System (Bio-Rad Laboratories, Hemel Hempstead, UK).

Glucose

Measurement of glucose was undertaken using the glucose oxidase and 4-aminoantipyrine enzymatic colorimetric method. The increase in absorbance at 510 nm was measured using a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK).

Insulin

Measurement of insulin was undertaken using a sandwich ELISA method (Abcam, Cambridge, UK). Samples and standards were added to pre-coated micro-titre plates and peroxidase-conjugated secondary antibody was added to create an antigen complex. Substrate solution tretramethylbenzidine was then added following 60 minutes of incubation, followed by the stop solution. OD was determined at 450 nm using a microplate reader and the concentration calculated from the standard curve.

Homeostatic model assessment of beta cell function (HOMA-B)

The HOMA-B equation was used to estimate pancreatic beta cell function.[19]

$$\text{HOMA-B} = 20 \times \frac{\text{Insulin (mU/l)}}{\text{Glucose (mmol/l)}} - 3.5$$

Homeostatic model assessment of insulin resistance (HOMA-IR)

The HOMA-IR equation was used to estimate insulin resistance.[19]

$$\text{HOMA-IR} = \frac{\text{Insulin (mU/l)} \times \text{Glucose (mmol/l)}}{22.5}$$

3.10 OTHER BIOMARKERS

Liver function tests

Measurements of alanine transaminase (ALT), aspartate transaminase (AST) and gamma-glutamyl transferase (GGT), were undertaken on a Randox daytona+ analyser using reagents from Randox Laboratories (Crumlin, UK).

Non-alcoholic fatty liver disease (NAFLD)-liver fat score

The NAFLD-liver fat score was used as a non-invasive tool for estimation of hepatic fat content which utilises presence of metabolic syndrome, presence of type 2 diabetes, fasting insulin, AST and ALT levels as predicting variables.[20]

NAFLD-liver fat score = -2.89 + 1.18 x Metabolic Syndrome (Y:1, N:0) + 0.45 x Type 2 Diabetes (Y:1, N:0) + 0.15 x Insulin (mU/L) + AST (U/L) – 0.94 x AST / ALT

Adiponectin

Measurement of adiponectin was undertaken using solid phase sandwich ELISA method (R&D Systems, Abingdon, UK). 50 µl of samples and standards were added to pre-coated microplate wells and incubated for 2 hours at room temperature. 200 µl of human adiponectin conjugate was then added followed by a further 2 hours of incubation. 200 µl of substrate solution was then added followed by 50 µl of stop solution after 30 minutes. The OD was determined using a microplate reader at 450 nm and the concentration calculated from the standard curve. The intra- and inter-assay CV were 3.5% and 6.5% respectively.

Leptin

Measurement of leptin was undertaken using solid phase sandwich ELISA method (R&D Systems, Abingdon, UK). 100 µl of samples and standards were added to pre-coated microplate wells and incubated for 2 hours at room temperature. 200 µl of human leptin conjugate was then added followed by a further 1 hour of incubation. 200 µl of substrate solution was then added followed by 50 µl of stop solution after 30 minutes. The OD was determined using a microplate reader at 450 nm and the concentration calculated from the standard curve. The intra- and inter-assay CV were 3.2% and 4.4% respectively.

Resistin

Measurement of resistin was undertaken using solid phase sandwich ELISA method (R&D Systems, Abingdon, UK). 100 µl of samples and standards were added to pre-coated microplate wells and incubated for 2 hours at room temperature. 200 µl of human resistin conjugate was then added followed by a further 2 hours of incubation. 200 µl of substrate solution was then added followed by 50 µl of stop solution after 30 minutes. The OD was determined using a microplate reader at 450 nm and the concentration calculated from the standard curve. The intra- and inter-assay CV were 4.7% and 8.4% respectively.

Anti-apolipoprotein A-I IgG

Measurements of anti-apoA-I IgG levels and positivity were undertaken at and using methods establish at the Department of Genetics and Laboratory Medicine, Geneva University Hospital (Geneva, Switzerland).[21-23]

Maxisorb plates (Nunc, Glostrup, Denmark) were coated with purified human-derived delipidated apoA-1 and then blocked with 2% BSA in PBS at 37°C. Samples diluted to 1:50 were added to coated and un-coated wells to allow assessment of individual non-specific binding. Alkaline phosphatase-conjugated anti-human IgG signal antibody (Sigma-Aldrich, St. Louis, MO, USA) was then added to each well and incubated for 1 hour, followed by 50 µl of phosphatase substrate *p*-nitrophenyl phosphate disodium (Sigma-Aldrich, St Louis, MO, USA) dissolved in diethanolamine buffer (pH 9.8). Each sample was tested in duplicates and the OD was determined at 405nm (Molecular Devices^{TM} Versa Max, Sunny Vale, CA, USA), with the corresponding non-specific binding subtracted from mean absorbance.

The upper reference range was derived from the 97.5th percentile of the reference population consisting 140 healthy blood donors which corresponded with an OD cut off of 0.64. An index consisting the ratio between sample OD and the positive control OD, expressed as a percentage, is further calculated to minimise the impact of inter-assay variation. The index value of 37% corresponded with the 97.5th percentile of

the normal distribution. Results with an absorbance value greater than 0.64 OD and an index value 37% or greater were considered positive for elevated anti-apoA-I IgG.

Androgen profile

Measurement of androgen profile was undertaken at Tallaght Hospital (Dublin, Ireland). Sex hormone binding globulin (SHBG) and DHEAS were measured using standard chemiluminescence immunoassays with assay CV of less than 5%. Androstenedione was measured using radioimmunoassay with assay CV of less than 5%.

3.11 STATISTICAL ANALYSES

This section provides an overview on the statistical analyses undertaken in this study. Further detailed descriptions of statistical analyses performed are provided within individual results chapters.

Statistical analyses were performed using SPSS for Mac (Version 23.0, IBM SPSS Statistics, Armonk, New York, USA) and figures produced using GraphPad Prism for Mac (Version 8.00, GraphPad Software, La Jolla California, USA). Continuous variables were assessed for normality using the Shapiro-Wilk test and visualisation of histograms. Results were generally presented as mean and standard deviation for parametric and mean and interquartile range for non-parametric variables.

For chapter 4, comparison of variables between baseline and post-intervention was undertaken using the paired sample t-test for parametric and the Wilcoxon signed-rank test for non-parametric variables. A *P*-value of less than 0.05 was considered to be statistically significant. Correlations between variables were assessed using Spearman's analyses with *P*-values of less than 0.01 being consider to be statistically significant due to multiple comparisons.

For chapter 5, non-parametric variables were normalised prior to analysis using nature logarithmic transformation. One-way univariate repeated measures ANOVA was performed to evaluate the effect of time on clinical characteristics and HDL-associated miRNA levels, with time as the within subject factor. Participants with missing miRNA data were excluded from the analysis. As there were a substantial number of participants with missing clinical characteristics at 6 months after surgery, clinical characteristics at baseline were compared to those at 6 and 12 months after surgery using individual paired samples t-test. Multiple testing correction was performed by Bonferroni correction for the two time-points, in which the threshold of *P*-value for significance was less than 0.025. Correlations between different HDL-associated miRNAs, as well as between changes in individual miRNAs and changes in other variables were performed using Spearman's test.

For chapter 6, comparison of variables between groups was undertaken using the independent samples t-test for parametric and the Mann-Whitney U test for non-parametric variables. Patients with PCOS were sub-divided into three groups based on tertiles of insulin resistance estimated using the HOMA-IR equation. Correlations between variables were assessed using bivariate Spearman's analyses. A *P*-value of less than 0.05 was considered to be statistically significant.

For chapter 7, presence of significant carryover effects was determined prior to assessment of treatment effect. Comparison of variables and treatment effects between ERN/LRP and placebo were performed using independent samples t-test for parametric distribution and Mann-Whitney U test for non-parametric distribution. Correlations between variables were assessed using Spearman's analyses. A *P*-value of less than 0.05 was considered to be statistically significant.

3.12 REFERENCES

1. Stegenga H, Haines A, Jones K, Wilding J, Guideline Development G. Identification, assessment, and management of overweight and obesity: summary of updated NICE guidance. *BMJ.* 2014;349:g6608.

2. Dunaif A. *Polycystic ovary syndrome*. Boston

St. Louis, Mo.: Blackwell Scientific Publications ;

Distributors, USA and Canada, Mosby-Year Book; 1992.

3. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem.* 1972;18:499-502.

4. Tsimikas S, Lau HK, Han KR, et al. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation.* 2004;109:3164-3170.

5. Tsimikas S, Fazio S, Viney NJ, Xia S, Witztum JL, Marcovina SM. Relationship of lipoprotein(a) molar concentrations and mass according to lipoprotein(a) thresholds and apolipoprotein(a) isoform size. *J Clin Lipidol.* 2018;12:1313-1323.

6. Taleb A, Witztum JL, Tsimikas S. Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark Med.* 2011;5:673-694.

7. Leibundgut G, Arai K, Orsoni A, et al. Oxidized phospholipids are present on plasminogen, affect fibrinolysis, and increase following acute myocardial infarction. *J Am Coll Cardiol.* 2012;59:1426-1437.

8. Arai K, Orsoni A, Mallat Z, et al. Acute impact of apheresis on oxidized phospholipids in patients with familial hypercholesterolemia. *J Lipid Res.* 2012;53:1670-1678.

9. Tsimikas S, Willeit P, Willeit J, et al. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J Am Coll Cardiol.* 2012;60:2218-2229.

10. Tsimikas S, Kiechl S, Willeit J, et al. Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. *J Am Coll Cardiol.* 2006;47:2219-2228.

11. Bertoia ML, Pai JK, Lee JH, et al. Oxidation-specific biomarkers and risk of peripheral artery disease. *J Am Coll Cardiol.* 2013;61:2169-2179.

12. Rodenburg J, Vissers MN, Wiegman A, et al. Oxidized low-density lipoprotein in children with familial hypercholesterolemia and unaffected siblings: effect of pravastatin. *J Am Coll Cardiol.* 2006;47:1803-1810.

13. Kiechl S, Willeit J, Mayr M, et al. Oxidized phospholipids, lipoprotein(a), lipoprotein-associated phospholipase A2 activity, and 10-year cardiovascular outcomes: prospective results from the Bruneck study. *Arterioscler Thromb Vasc Biol.* 2007;27:1788-1795.

14. Tsimikas S, Mallat Z, Talmud PJ, et al. Oxidation-specific biomarkers, lipoprotein(a), and risk of fatal and nonfatal coronary events. *J Am Coll Cardiol.* 2010;56:946-955.

15. Tsimikas S, Witztum JL, Miller ER, et al. High-dose atorvastatin reduces total plasma levels of oxidized phospholipids and immune complexes present on apolipoprotein B-100 in patients with acute coronary syndromes in the MIRACL trial. *Circulation.* 2004;110:1406-1412.

16. Ravandi A, Boekholdt SM, Mallat Z, et al. Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study. *J Lipid Res.* 2011;52:1829-1836.

17. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol.* 2010;30:796-801.

18. Tabet F, Cuesta Torres LF, Ong KL, et al. High-Density Lipoprotein-Associated miR-223 Is Altered after Diet-Induced Weight Loss in Overweight and Obese Males. *PLoS One.* 2016;11:e0151061.

19. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* 1985;28:412-419.

20. Kotronen A, Peltonen M, Hakkarainen A, et al. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology.* 2009;137:865-872.

21. Vuilleumier N, Rossier MF, Pagano S, et al. Anti-apolipoprotein A-1 IgG as an independent cardiovascular prognostic marker affecting basal heart rate in myocardial infarction. *Eur Heart J.* 2010;31:815-823.

22. Montecucco F, Vuilleumier N, Pagano S, et al. Anti-Apolipoprotein A-1 auto-antibodies are active mediators of atherosclerotic plaque vulnerability. *Eur Heart J.* 2011;32:412-421.

23. Antiochos P, Marques-Vidal P, Virzi J, et al. Anti-Apolipoprotein A-1 IgG Predict All-Cause Mortality and Are Associated with Fc Receptor-Like 3 Polymorphisms. *Front Immunol.* 2017;8:437.

CHAPTER 4: EFFECT OF BARIATRIC SURGERY ON PLASMA LEVELS OF OXIDISED PHOSPHOLIPIDS, BIOMARKERS OF OXIDISED LDL, AND LIPOPROTEIN(A)

Published in Journal of Clinical Lipidology

Author's contribution:

The author undertook patient recruitment, study visit assessments, and processing of blood samples for storage. The author also undertook laboratory analyses under supervision apart from the OxPL-apoB and Lp(a) assays which were undertaken at the University of California San Diego. The author analysed and interpreted the data. He undertook research of available literature, wrote the first draft, and produced the final manuscript with Professor Sotirios Tsimikas and Dr Handrean Soran.

Effect of bariatric surgery on plasma levels of oxidised phospholipids, biomarkers of oxidised LDL and lipoprotein(a)

Jan Hoong Ho[1,2], Safwaan Adam[1,2], Yifen Liu[2], Shazli Azmi[2], Shaishav Dhage[1,2], Akheel A Syed[3], Basil J Ammori[4], Rachelle Donn[2], Adrian Heald[3], Martin J Gibson[3], Rayaz A Malik[2,5], Xiaohong Yang[6], Paul N Durrington[2], Sotirios Tsimikas[6], Handrean Soran[1,2]

[1]Department of Medicine, Manchester University NHS Foundation Trust, Manchester, UK
[2]Lipid Research Group, The University of Manchester, Manchester, UK
[3]Department of Diabetes & Endocrinology, Salford Royal NHS Foundation Trust, Salford, UK
[4]Department of Surgery, Salford Royal NHS Foundation Trust, Salford, UK
[5]Weill Cornell Medicine-Qatar, Doha, Qatar
[6]Sulpizio Cardiovascular Center, Division of Cardiovascular Medicine, University of California, San Diego, USA

*Co-corresponding authors:

Prof Sotirios Tsimikas
Director of Vascular Medicine and Professor of Medicine
Email: stsimikas@health.ucsd.edu

Dr Handrean Soran MSc, MD, FRCP
Consultant Physician and Endocrinologist
E-mail: hsoran@aol.com; handrean.soran@mft.nhs.uk

4.1 ABSTRACT

Background and aims: Obesity is associated with adverse cardiovascular outcomes and this is improved following bariatric surgery. Oxidised phospholipids (OxPL) are thought to reflect the pro-inflammatory effects of lipoprotein(a) [Lp(a)], and both are independent predictors of cardiovascular disease. Our study sought to determine the impact of bariatric surgery on OxPL, biomarkers of oxidised LDL (OxLDL) and Lp(a).

Methods: This is a prospective, observational study of 59 patients with severe obesity undergoing bariatric surgery. Blood samples were obtained prior to surgery and at 6 and 12 months after. Sixteen patients attending the tertiary medical weight management clinic at the same centre were also recruited for comparison. Lipid and metabolic blood parameters, OxLDL, OxPL on apolipoprotein B-100 (OxPL-apoB), IgG and IgM autoantibodies to MDA-LDL, IgG and IgM apoB-immune complexes and Lp(a) were measured.

Results: Reduction in body mass index (BMI) was significant following bariatric surgery, from median 48 kg/m^2 at baseline to 37 kg/m^2 at 6 months and 33 kg/m^2 at 12 months. OxPL-apoB levels decreased significantly at 12 months following surgery [5.0 (3.2–7.4) to 3.8 (3.0–5.5) nM, *P*=0.001], while contrastingly, Lp(a) increased significantly [10.2 (3.8–31.9) to 16.9 (4.9–38.6) mg/dl, *P*=0.002]. There were significant post-surgical decreases in IgG and IgM biomarkers, particularly at 12 months, while OxLDL remained unchanged.

Conclusions: Bariatric surgery results in a significant increase in Lp(a) but reductions in OxPL-apoB and other biomarkers of oxidised lipoproteins, suggesting increased synthetic capacity and reduced oxidative stress. These biomarkers might be clinically useful to monitor physiological effects of weight loss interventions.

KEYWORDS

Obesity, bariatric surgery, weight reduction, lipoprotein(a), oxidised phospholipids, autoantibodies to oxidised LDL, apoB immune complexes.

4.2 INTRODUCTION

Obesity is an independent risk factor for cardiovascular disease (CVD). It is a continuum of metabolic syndrome and is associated with chronic low-grade inflammation and increased systemic oxidative stress.[1, 2] Oxidative stress and inflammation are closely related and are central to the atherosclerotic process.[3] Increased oxidative stress results in oxidative modification of low-density lipoprotein (LDL) and other lipoproteins and subsequent generation of proinflammatory and proatherogenic oxidation-specific epitopes (OSE), such as oxidised phospholipids (OxPL) and malondialdehyde epitopes on LDL.[4] Autoantibodies and immune complexes against oxidation-specific epitopes reflect the immune consequences of OSE acting as danger associated molecular patterns and provide an indirect measure of lipoprotein-associated oxidation.[5]

OxPL are key contributors to the atherosclerotic process and are involved in inflammatory cascade activation and plaque destabilisation.[6] Among lipoproteins lipoprotein(a) [Lp(a)] preferentially binds OxPL and is also the main lipoprotein carrier for OxPL.[4, 7] OxPL can be measured on apolipoprotein B-100 (apoB) containing lipoproteins (OxPL-apoB). Since the majority of OxPL on lipoproteins are on Lp(a), which is an apoB-containing lipoprotein, OxPL-apoB and Lp(a) are usually correlated in plasma. OxPL present on Lp(a) are bound covalently to apolipoprotein(a) and also present in the lipid phase of apoB. Lp(a) and OxPL-apoB levels are correlated in cross-sectional studies[8-10] with correlation coefficients ranging from r=0.14 to 0.83 depending on the populations studied.[11] Both Lp(a) and OxPL-apoB are independent predictors of CVD,[7, 12-17] however, OxPL-apoB often remains an independent predictor when evaluated with Lp(a) in multivariate adjustment models, suggesting a broader reflection of risk than simply measuring plasma levels of Lp(a).[7, 18]

Although elevated Lp(a) levels are not a feature of obesity, studies have found Lp(a) levels to be inversely associated with insulin resistance and insulin levels.[19-21] A rise in Lp(a) levels have also been demonstrated following dietary-induced weight loss,[22] particularly in response to low-fat diets.[23, 24] Bariatric surgery produces a sustained improvement in conventional CVD risk factors[25, 26] and a reduction in cardiovascular events and death.[27-30] Its effect on Lp(a), OSE and other biomarkers of oxidised

lipoproteins, however, are yet to be established. In this study, we sought to evaluate the effect of bariatric surgery on Lp(a), OxPL-apoB, oxidised LDL (OxLDL), autoantibodies to malondialdehyde-modified LDL (MDA-LDL) and apoB immune complexes (apoB-IC).

4.3 MATERIALS AND METHODS

Study design and participants

This is a prospective observational study of 59 patients with severe obesity undergoing metabolic surgery at the Salford Royal NHS Foundation Trust tertiary weight management centre (Salford, UK). Sixteen patients attending the tertiary medical weight management clinic at the same centre were recruited as control patients. Patients with anaemia, acute coronary syndrome within 6 months, history of malignancy and chemotherapy, active infections, immunological or haematological disorders, human immunodeficiency virus (HIV), chronic obstructive airway disease, current steroid therapy, receiving immunotherapy or monoclonal antibodies and autoimmune diseases were excluded. Study assessments were undertaken at the National Institute of Health Research/Wellcome Trust Clinical Research Facility (Manchester, UK) at baseline, 6 months, and 12 months after bariatric surgery. Control participants were assessed at baseline and 12 months. This study has approval from the Greater Manchester Central Research and Ethics Committee and study assessments were in accordance with the 1964 Helsinki declaration. Written informed consent was obtained from all patients prior to participation in this study.

Serum and plasma samples

Venous blood samples were obtained from patients between 0900 to 1100 after an overnight fast of at least 12 hours. Serum and EDTA-plasma were isolated by centrifugation at 2000 x *g* for 15 minutes at 4°C within 2 hours of collection. Aliquots were stored frozen at -80°C until biochemical analyses performed at the end of study. Prior studies have shown that oxidative biomarkers have acceptable within-

person 5-year reproducibility of frozen samples (correlation coefficient 0.78) and are stable over 24 h on ice (intra-class correlation coefficient 0.96).[31]

Laboratory analyses

Total cholesterol was measured using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase method, triglycerides by the glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase method, and apoB was assayed using immunoturbidimetric assays (ABX Diagnostics, Shefford, UK). High-density lipoprotein cholesterol (HDL-C) was assayed using a second-generation homogenous direct method (Roche Diagnostics, Burgess Hill, UK). All these tests were performed on a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK). The laboratory participated in the RIQAS (Randox International Quality Assessment Scheme; Randox Laboratories, Dublin, Ireland) scheme which is CRC calibrated. Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formula. Proprotein convertase subtilisin/kexin 9 (PCSK9) levels were measured at the research laboratory of University of California San Diego using a validated in-house ELISA as previously described.[32]

Alanine transaminase (ALT), aspartate transaminase (AST) and gamma-glutamyl transferase (GGT), were measured using assays from Randox (Randox Laboratories, Crumlin, UK), following standardised procedures in accordance with the International Federation of Clinical Chemistry (IFCC). Hepatic fat was estimated using the NAFLD-liver fat score.[33]

Glycosylated haemoglobin (HbA1c) was measured using standard laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust (Manchester, UK). Glucose and insulin were measured using Abcam ELISA kits (Abcam, Cambridge, UK) and used to calculate homeostatic model assessment of insulin resistance (HOMA-IR) and beta cell function (HOMA-B).[34]

Lipoprotein(a), oxidised phospholipids, OxLDL, apolipoprotein B-100 immune complexes and MDA-LDL autoantibody titres

Lp(a) levels were measured by a validated chemiluminescent ELISA with monoclonal antibody LPA4 as previously described.[9]

OxPL-apoB was measured with a chemiluminescent immunoassay using murine monoclonal antibody E06.[4, 35-37] E06 recognizes the phosphocholine (PC) group on oxidised but not native phospholipids and similarly recognises the PC covalently bound to bovine serum albumin (BSA) in PC-BSA. A 1:50 dilution of plasma in 1% BSA in Tris-buffered saline (TBS) was added to microtiter wells coated with the apoB specific monoclonal antibody MB47. A saturating amount of apoB was added to each well and therefore equal numbers of apoB particles are captured in each well. Biotinylated E06 was then added to determine the content of OxPL-apoB. The values are reported as nanomolar (nM) PC-OxPL using a standard curve of nM PC equivalents as previously described.[31] The OxPL-apoB value reflects the absolute content of OxPL per a constant amount of captured apoB lipoprotein and is independent of plasma levels of apoB or LDL-C. The coefficient of variation of the Lp(a) and OxPL-apoB assays are 6.0 to 7.4% and 6.0 to 10.0% respectively.

Chemiluminescence ELISAs were used to measure IgG and IgM autoantibodies to MDA-LDL, and IgG and IgM apoB-IC as previously described.[5, 38] For IgG and IgM autoantibodies to malondialdehyde modified LDL (MDA)-LDL, a 1:200 dilution of plasma was added to microtitre wells coated with MDA-LDL (5μg/ml) and binding to MDA-LDL was detected using alkaline phosphatase labeled goat anti human IgG and IgM (Sigma). ApoB-ICs were detected by plating murine monoclonal antibody MB47 to bind a saturating amount of human apoB, plasma (1:50 dilution) was added and IgG or IgG autoantibodies binding to the captured apoB (i.e., apoB-IC) were detected with alkaline phosphatase labeled goat anti human IgG or IgM as above.[5, 38]

OxLDL was measured in plasma using Mercodia ELISA kits (Diagenics Ltd, Milton Keynes, UK).

Statistical analyses

Statistical analyses were performed using SPSS for Mac (Version 23.0, IBM SPSS Statistics, Armonk, New York, USA) and figures were produced using GraphPad Prism for Mac (Version 8.00, GraphPad Software, La Jolla California, USA). Comparison of variables between baseline and post-intervention was undertaken using the paired sample t-test for parametric and the Wilcoxon signed-rank test for non-parametric variables. A *P*-value of less than 0.05 was considered to be statistically significant. Normality of data distribution was assessed using the Shapiro-Wilk test and visualising of histograms. Correlations between variables were assessed using Spearman's analyses with *P*-values of less than 0.01 being consider to be statistically significant due to multiple comparisons. Results are presented as mean and standard deviation for parametric or as median and interquartile range for non-parametric variables.

4.4 RESULTS

Baseline characteristics and changes after bariatric surgery and medical weight management

The baseline characteristics of patients who underwent bariatric surgery and medical weight management are shown in Table 4.1. Within the surgically-treated group, 31 (53%) had Roux-en-Y gastric bypass (RYGB), 19 (32%) sleeve gastrectomy, and 9 (15%) omega loop bypass. The mean age was 50.1 years and the median BMI was 48 kg/m^2. The prevalence of type 2 diabetes was 63% (75% including patients with pre-diabetes). Thirty percent of the cohort were statin-treated and this is unchanged following surgery. The medically-treated group had a higher mean age of 62.5 years and a lower median BMI of 36 kg/m^2. All patients had pre-existing type 2 diabetes and were statin-treated. HbA1c (*P*=0.356), fasting glucose (*P*=0.253), fasting insulin (*P*=0.361), and HOMA-IR (*P*=0.088) were lower in the medically-treated group but not statistically significant. Total cholesterol (*P*=0.182), triglyceride (*p*=0.859), LDL-C (*P*=0.246), and NAFLD-liver fat score (*P*=0.769) were not significantly different between the two groups; while HDL-C (*P*=0.007), apoB (*p*=0.769), Lp(a) (*P*<0.001), and OxLDL (*P*=0.020) were lower, and CRP (*P*=0.002) and OxPL-apoB (*P*=0.009) higher in the surgical group (Table 4.1, Table 4.2 and Table 4.3).

Changes in clinical characteristics after bariatric surgery and medical weight management

As expected, there was significant reduction in median body mass index (BMI) from 48 (44–52) kg/m^2 at baseline to 37 (33–40) kg/m^2 at 6 months and 33 (30–37) kg/m^2 at 12 months after surgery (Table 4.2). There were also significant reductions in markers of glycaemia (HbA1c, fasting glucose, fasting insulin, HOMA-IR, and HOMA-B) and markers of liver function and liver fat (ALT, GGT, and NAFLD-liver fat score) following surgery (Table 4.2). In comparison, a slight reduction in median BMI from 36 (27–41) kg/m^2 to 33 (27–40) kg/m^2 was achieved following medical weight management but no changes in markers of glycaemia, markers of liver function and fat, and lipid profile (Table 4.2 and 4.3).

Table 4.1. Baseline characteristics of patients who underwent bariatric surgery and medical weight management.

	Bariatric surgery (n=59)	**Medical weight management (n=16)**
Clinical characteristics		
Age, y	50.1 (10.0)	62.5 (12.2)
Female, n (%)	41 (69)	10 (63)
Ethnicity		
Caucasian, n (%)	55 (93)	14 (88)
South Asian, n (%)	4 (7)	2 (12)
Procedure		
RYGB, n (%)	31 (53)	
Sleeve gastrectomy, n (%)	19 (32)	
Omega loop bypass, n(%)	9 (15)	
Type 2 diabetes, n (%)	37 (63)	16 (100)
Pre-diabetes & type 2 diabetes, n (%)	44 (75)	16 (100)
Statin, n (%)	30 (51)	16 (100)
BMI, kg/m^2	48 (44–52)	36 (27–41)
HbA1c, mmol/mol	48 (39–58)	44 (42–46)
eGFR, mL/min/1.73 m^2	127 (30)	110 (29)
CRP, mg/L	7.5 (3.7–13.6)	2.1 (1.7–6.1)
Fasting glucose, mmol/L	6.6 (5.6–8.3)	5.7 (4.8–6.1)
Fasting insulin, mU/L	18.4 (10.9–37.0)	12.1 (8.1–24.6)
HOMA-IR	6.3 (3.3–9.9)	3.2 (2.1–6.0)
HOMA-B	108.1 (56.7–253.2)	109.5 (90.8–530.4)

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables.

Abbreviations: BMI, body mass index; CRP, C-reactive protein; HOMA-B, homeostatic model assessment for beta cell function; HbA1c, glycosylated haemoglobin; HOMA-IR, homeostatic model assessment for insulin resistance.

Table 4.2. Changes in clinical characteristics and markers of glycemia following bariatric surgery and medical weight management.

	Bariatric surgery (n=59)			Medical weight management (n=16)	
	Baseline	**6 months**	**12 months**	**Baseline**	**12 months**
BMI, kg/m²	48 (44–52)	**37 (33–40)****	**33 (30–37)****	36 (27–41)	**33 (27–40)***
HbA1c, mmol/mol	48 (39–58)	**36 (34–43)****	**35 (33–40)****	44 (42–46)	41 (40–46)
Fasting glucose, mmol/L	6.6 (5.6–8.3)	6.2 (5.0–7.5)	**5.8 (5.0–6.8)****	5.7 (4.8–6.1)	5.4 (5.0–6.6)
Fasting insulin, mU/L	18.4 (10.9–37.0)	**8.3 (5.5–17.9)***	**8.0 (4.8–16.6)****	12.1 (8.1–24.6)	16.4 (7.5–25.3)
HOMA-IR	6.3 (3.3–9.9)	**2.6 (1.4–6.0)***	**2.0 (1.0–4.4)****	3.2 (2.1–6.0)	3.7 (1.7–8.9)
HOMA-B	108.1 (56.7–253.2)	**64.7 (36.0–128.2)***	**77.9 (41.7–149.2)***	109.5 (90.8–530.4)	162.3 (87.3–209.8)
eGFR, mL/min/1.73 m²	127 (30)	119 (27)	**120 (27)****	110 (29)	**105 (29)****
CRP, mg/L	7.5 (3.7–13.6)	**1.0 (0.4–2.1)****	**1.4 (0.5–4.0)****	2.1 (1.7–6.1)	2.2 (1.6–5.5)
ALT, U/L	7.0 (5.2–10.7)	5.3 (3.0–10.7)	**5.4 (3.7–8.2)****	5.5 (4.0–11.3)	5.6 (5.1–8.5)
AST, U/L	10.6 (6.5–19.3)	10.2 (6.1–19.9)	10.8 (8.2–17.3)	8.2 (5.7–11.9)	8.7 (6.1–11.6)
GGT, U/L	33.1 (24.0–52.6)	**20.3 (15.5–28.4)****	**21.2 (12.6–32.0)****	26.9 (19.9–43.2)	29.5 (20.2–47.0)
NAFLD-Liver fat score	0.24 (-1.0 –2.6)	**-2.05 (-3.83–0.62)****	**-2.56 (-3.75–-0.66)****	-0.0 (-1.5–2.4)	-0.05 (-0.56–2.47)

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables. Comparison of variables post-intervention with baseline was performed using paired t-test for parametric and Wilcoxon signed-rank test for non-parametric variables. **P*<0.05, ***P*<0.01

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase, BMI, body mass index; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyl transferase; HOMA-B, homeostatic model assessment for beta cell function; HbA1c, glycosylated haemoglobin; HOMA-IR, homeostatic model assessment for insulin resistance; NAFLD, non-alcoholic fatty liver disease.

Table 4.3. Changes in lipid profile and markers of lipoprotein-associated oxidative stress following bariatric surgery and medical weight management.

	Bariatric surgery (n=59)			Medical weight management (n=16)	
	Baseline	**6 months**	**12 months**	**Baseline**	**12 months**
Total cholesterol, mmol/L	4.1 (3.6–5.1)	4.1 (3.5–4.8)	4.3 (3.8–5.1)	4.8 (1.3)	4.9 (1.2)
Triglyceride, mmol/L	1.5 (1.1–2.0)	**1.2 (0.9–1.6)****	**1.3 (0.9–1.6)****	1.4 (1.0–2.4)	1.4 (1.0–2.2)
HDL-C, mmol/L	0.94 (0.84–1.13)	**1.11 (0.92–1.26)***	**1.18 (1.00–1.36)****	1.25 (1.06–1.57)	1.36 (1.08–1.63)
LDL-C, mmol/L	2.5 (1.9–3.3)	2.3 (1.8–3.1)	2.5 (2.0–3.2)	2.9 (1.0)	2.8 (1.1)
ApoB, mg/dL	70 (60–84)	**67 (54–83)***	**62 (57–78)****	95 (30)	102 (27)
Lp(a), mg/dL	10.2 (3.8–31.9)	11.5 (4.0–28.8)	**16.9 (4.9–38.6)****	44.4 (26.9–74.7)	43.2 (29.9 –71.1)
PCSK9, ng/mL	162.2 (80.8–271.2)	154.6 (59.4–253.6)	153.5 (70.8–243.4)	353.8 (213.9–460.7)	437.1 (214.6–552.5)
OxLDL, ng/mL	52.4 (34.1–129.8)	55.3 (34.6–153.4)	65.8 (34.2–130.6)	113.8 (59.6–155.3)	**148.4 (66.9–167.3)***
OxPL-apoB, nM	5.0 (3.2–7.4)	3.9 (3.3–5.6)	**3.8 (3.0–5.5)****	2.7 (1.9–5.3)	4.3 (2.1–7.8)
OxPL-apoB to Lp(a) ratio	0.57 (0.23–1.03)	**0.39 (0.20–0.78)***	**0.27 (0.14–0.69)****	0.05 (0.04–0.11)	0.06 (0.05–0.14)
IgG MDA-LDL, RLU	16723 (11800–26413)	**13548 (8873–20887)****	**11710 (8873–15896)****	11253 (8418–25891)	12488 (7495–26032)
IgM MDA-LDL, RLU	32633 (21508–50824)	**25168 (16174–41359)****	**26664 (15675–39586)****	60017 (49565–82378)	**56510 (45988–60801)***
IgG IC, RLU	33367 (20074–42552)	**21366 (15658–30215)****	**17755 (10448–24554)****	17324 (5340)	15585 (6759)
IgM IC, RLU	19875 (14772–27179)	15084 (11920–27641)	**15410 (8890–20668)****	17626 (11133)	15335 (8002)

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables. Comparison of variables post-intervention with baseline was performed using paired t-test for parametric and Wilcoxon signed-rank test for non-parametric variables. **P*<0.05, ***P*<0.01

Abbreviations: HDL-C, high-density lipoprotein cholesterol; IC, immune complex; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; PCSK9, proprotein convertase subtilisin/kexin 9; RLU, reactive light units.

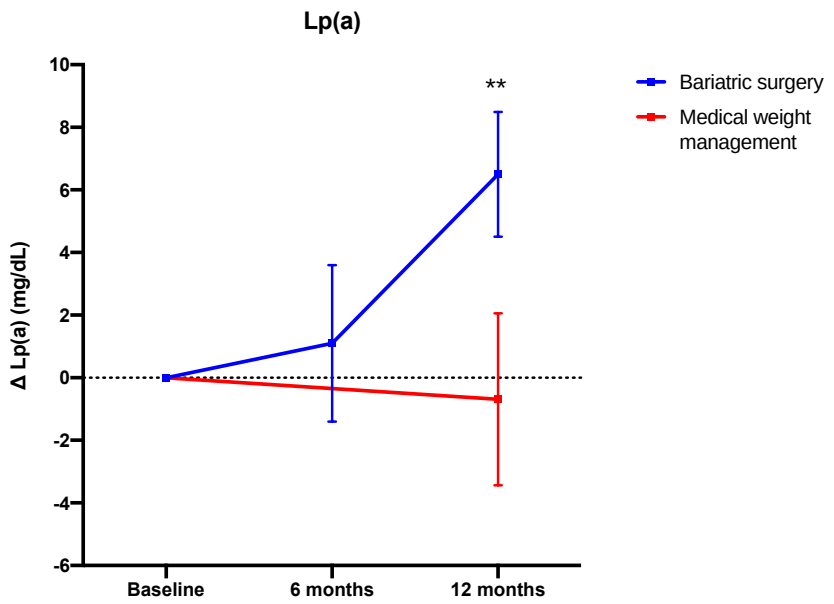
Changes in lipid profile, lipoprotein(a), OxLDL, and OxPL-apoB after bariatric surgery and medical weight management

Triglyceride and apoB levels decreased, and HDL-C increased significantly following surgery (Table 4.3). Lp(a) increased significantly at 12 months following bariatric surgery [10.2 (3.8–31.9) vs 16.9 (4.9–38.6) mg/dL, *P*=0.002] (Figure 4.1 and Table 4.3). There was no significant change following medical weight management.

In contrast to Lp(a), there was a trend for reduction of OxPL-apoB at 6 months [5.0 (3.2–7.4) vs 3.9 (3.3–5.6) nM, *P*=0.076] with further significant reductions at 12 months after surgery [5.0 (3.2–7.4) vs 3.8 (3.0–5.5) nM, *P*=0.001] (Figure 4.1 and 4.2, and Table 4.3). These changes were accompanied by a reduction in OxPL-apoB to Lp(a) ratio at both 6 months and 12 months after surgery [0.57 (0.23–1.03) vs 0.39 (0.20–0.78) vs 0.27 (0.14–0.69), *P*=0.035 and *P*<0.001 respectively]. No significant changes were observed following medical weight management.

Whilst there were no significant changes in OxLDL at both 6 and 12 months after bariatric surgery, a significant increase was observed following medical weight management [113.8 (59.6–155.3) vs 148.4 (66.9–167.3) ng/mL, *P*=0.028] (Table 4.3).

A.



B.

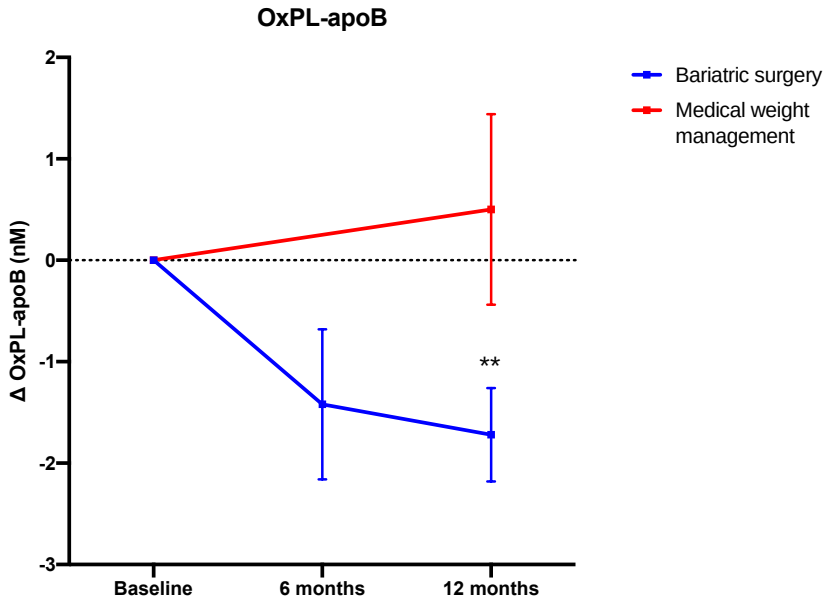
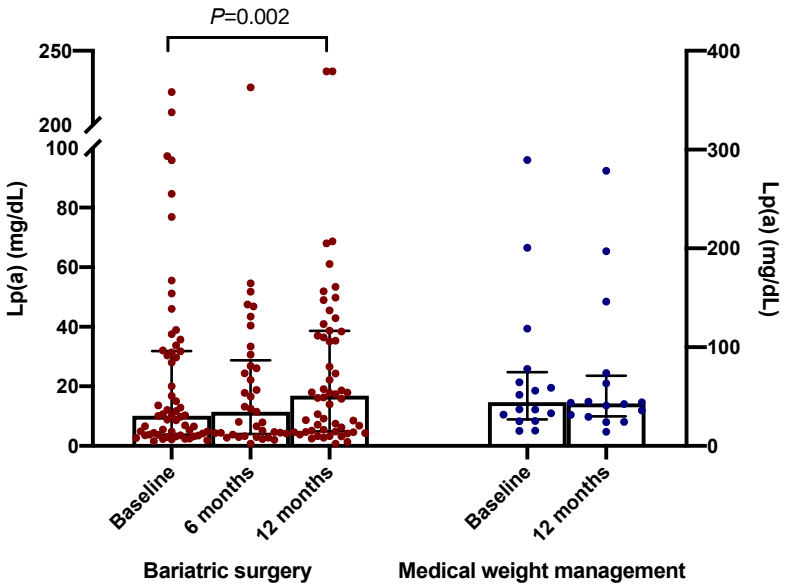


Figure 4.1. Absolute changes in lipoprotein(a) and OxPL-apoB following bariatric surgery and medical weight management.

Absolute changes in A) lipoprotein(a) and B) OxPL-apoB from baseline to 6 months and 12 months after bariatric surgery. Data are presented as mean and standard error of the mean. Comparisons of variables at 6 and 12 months with baseline were performed using paired samples t-test or parametric and Wilcoxon signed-rank test for non-parametric variables. *P<0.05, **P<0.01

A.



B.

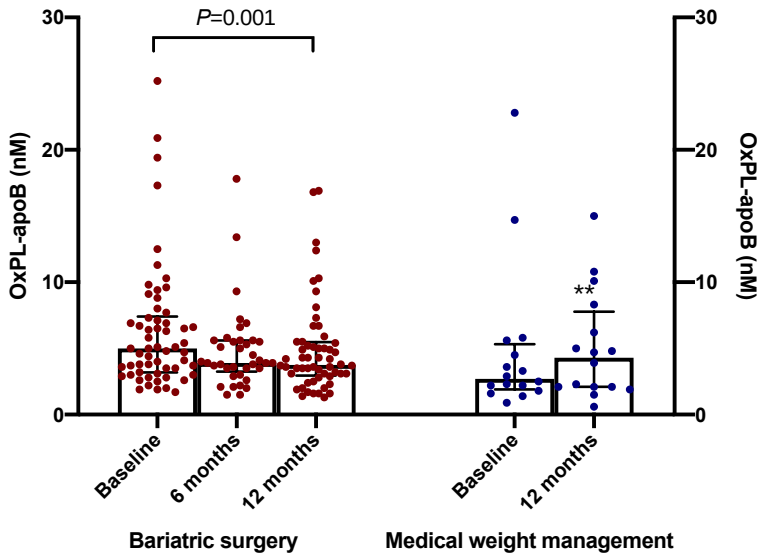


Figure 4.2. Comparison of lipoprotein(a) and OxPL-apoB levels at baseline and after bariatric surgery and medical weight management.

Levels of A) lipoprotein(a) and B) OxPL-apoB from baseline to 6 months and 12 months after bariatric surgery.

Data are presented as median and interquartile range. Comparisons of variables at 6 and 12 months with baseline were performed using paired samples t-test or parametric and Wilcoxon signed-rank test for non-parametric variables.

Changes in MDA-LDL autoantibodies and apolipoprotein B-100 immune complexes after bariatric surgery and medical weight management

Following bariatric surgery, there were significant reductions in IgG MDA-LDL [16723 (11800–26413) vs 13548 (8873–20887) RLU, *P*<0.001] and IgG apoB-IC [33367 (20074–42552) vs 21366 (15658–30215) RLU, *P*<0.001] at 6 months, with further reductions observed at 12 months [16723 (11800–26413) vs 11710 (8873–15896) RLU and 33367 (20074–42552) vs 17755 (10448–24554) RLU, both *P*<0.001] (Figure 4.3 and Table 4.3).

Similarly, IgM MDA-LDL were reduced significantly at both 6 months [32633 (21508–50824) vs 25168 (16174–41359) RLU, *P*=0.001] and 12 months [32633 (21508–50824) vs 26664 (15675–39586) RLU, *P*<0.001] after bariatric surgery. Significant reductions in IgM apoB-IC was also observed at 12 months after surgery [19875 (14772–27179) vs 15410 (8890–20668) RLU, *P*<0.001], with a trend for reduction at 6 months [19875 (14772–27179) vs 15084 (11920–27641) RLU, *P*=0.051] which did not achieve statistical significance.

In comparison, there was significant reduction in IgM MDA-LDL [60017 (49565–82378) vs 56510 (45988–60801) RLU, *p*=0.020] and a trend for reduction in IgM apoB-IC which did not achieve statistically significance [17626 (11133) vs 15335 (8002) RLU, *p*=0.085], but no significant changes in IgG MDA-LDL and IgG apoB-IC following medical weight management (Figure 4.3 and Table 4.3).

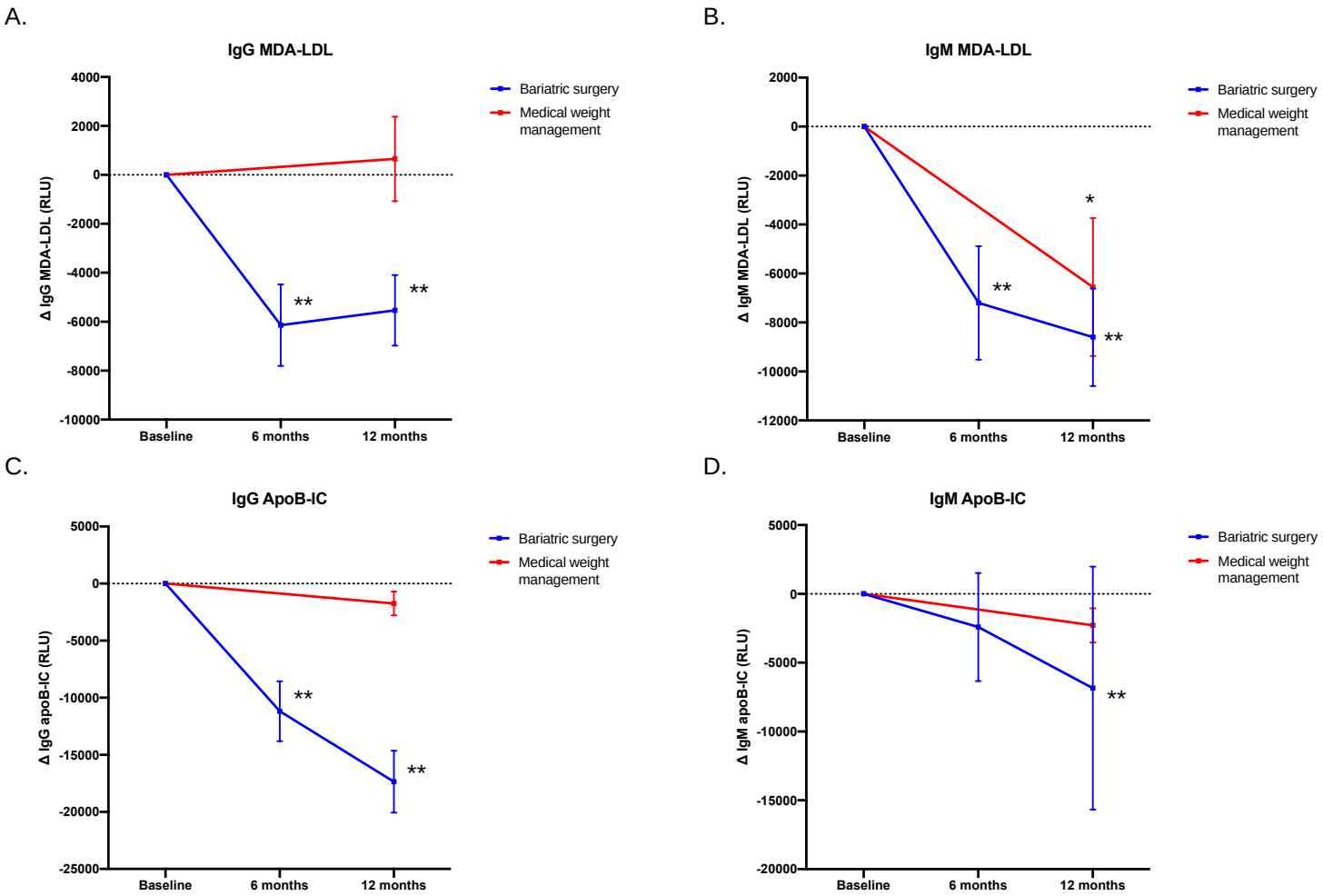


Figure 4.3. Absolute changes in IgG and IgM MDA-LDL and apoB-IC between bariatric surgery and medical weight management.

Absolute changes in A) IgG MDA-LDL, B) IgM MDA-LDL, C) IgG apoB-IC, and D) IgM apoB-IC from baseline to 6 months and 12 months after bariatric surgery. Data are presented as mean and standard error of the mean. Comparisons of variables at 6 and 12 months with baseline were performed using paired samples t-test for parametric and Wilcoxon signed-rank test for non-parametric variables. *P<0.05, **P<0.01

Correlations between biomarkers, clinical characteristics and lipid profile

Lp(a) correlated positively with OxPL-apoB, IgM MDA-LDL and OxLDL at baseline and 12 months (Table 4.4). IgG MDA-LDL correlated positively with IgG apoB-IC and both baseline and 12 months. IgM MDA-LDL correlated positively with IgM apoB-IC at 12 months but no baseline. There were no significant correlations between OxPL-apoB and OxLDL both at baseline and 12 months after surgery. While there was no significant correlation between Lp(a) and triglyceride at baseline (Table 4.5), change in Lp(a) did correlate negatively with change in triglyceride at 12 months after surgery (Figure 4.4 and Table 4.6).

There was a negative correlation was present between change in OxPL-apoB and change in triglyceride at 12 months after surgery (Table 4.6), which was maintained among patients with type 2 diabetes (Spearman's ρ=-0.463, *P*=0.007). Positive correlations in both baseline levels, and absolute changes in OxPL-apoB and PCSK9 were observed (Table 4.5 and 4.6).

OxLDL correlated positively with total cholesterol, HDL-C, and LDL-C in both baseline levels and absolute changes at 12 months after surgery. There was a positive correlation between change in OxLDL and change in apoB but a similar correlation was not observed at baseline (Table 4.5 and 4.6, and Figure 4.4).

The change in Lp(a) correlated negatively with changes in fasting glucose, fasting insulin, and HOMA-IR at 12 months after surgery (Table 4.7). The negative correlation with HOMA-IR was maintained in the sub-analysis of patients with type 2 diabetes (Spearman's ρ=-0.479, *P*=0.005). There was a trend of change in Lp(a) and change in NAFLD-liver fat score which did not achieve statistical significance (Spearman's ρ=-0.309, *P*=0.033) (Figure 4.4). Lp(a) correlated positively with AST at baseline (Table 4.5).

Similarly, there was a negative correlation between change in OxPL-apoB and change in NAFLD-liver fat score was observed at 12 months after surgery (Spearman's ρ=-0.384, *P*=0.004) (Table 4.7) which was maintained among patients with type 2 diabetes (Spearman's ρ=-0.530, *P*=0.003).

There were no significant correlations between change in CRP and changes in OxPL-apoB, OxLDL, and autoantibodies and immune complexes to MDA-LDL. Likewise, no significant correlations were noted between change in CRP and changes in NAFLD-liver fat score, fasting glucose, and HOMA-IR. There was, however, a positive correlation between change in CRP and fasting insulin (Spearman's ρ=0.285, *P*=0.003).

Table 4.4. Bivariate Spearman correlation between Lp(a) and markers of lipoprotein-associated oxidative stress at baseline and 12 months after surgery.

	Lp(a)	***OxPL-apoB***	***IgG MDA-LDL***	***IgM MDA-LDL***	***IgG apoB-IC***	***IgM apoB-IC***
Baseline						
Lp(a), mg/dL	-					
OxPL-apoB, nM	**0.432****	-				
IgG MDA-LDL, RLU	0.164	0.060	-			
IgM MDA-LDL, RLU	**0.348****	0.240	0.227	-		
IgG IC, RLU	-0.198	0.054	**0.530****	-0.230	-	
IgM IC, RLU	-0.221	0.213	0.117	0.196	0.322	-
oxLDL, ng/mL	**0.402****	0.042	-0.048	0.232	**-0.481****	**-0.464****
12 months						
Lp(a), mg/dL	-					
OxPL-apoB, nM	**0.490****	-				
IgG MDA-LDL, RLU	0.178	0.123	-			
IgM MDA-LDL, RLU	0.315	-0.007	0.260	-		
IgG IC, RLU	0.024	0.153	**0.514****	-0.001	-	
IgM IC, RLU	-0.035	0.143	0.090	**0.450****	0.139	-
OxLDL, ng/mL	0.290	-0.022	0.193	0.295	-0.259	-0.186

Abbreviations: IC, immune complex; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; RLU, reactive light units. ***P*<0.01

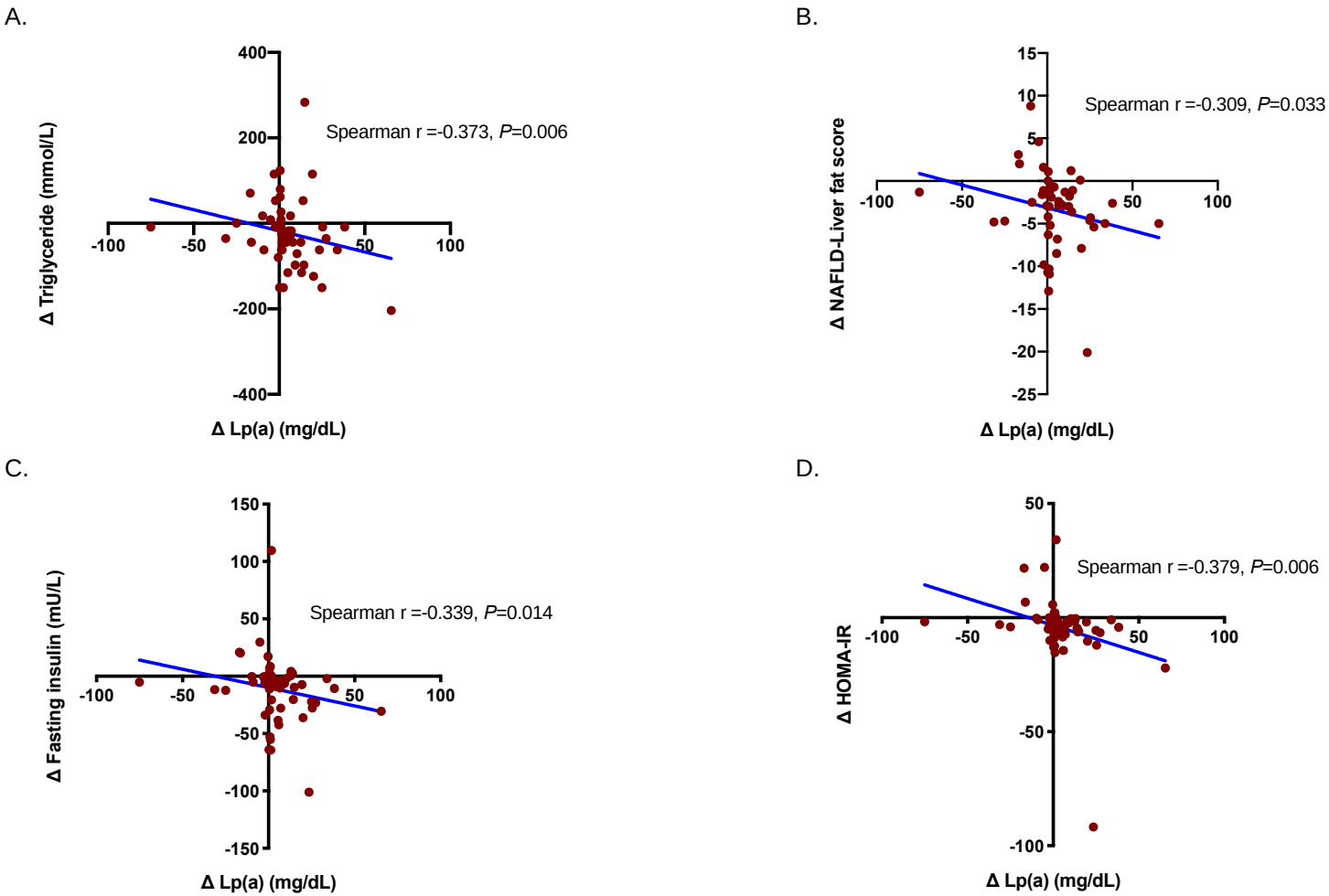


Figure 4.4. Correlations between changes in lipoprotein(a), triglyceride, and markers of insulin resistance and hepatic fat at 12 months after bariatric surgery.

Bivariate spearman correlation between change in lipoprotein(a) and changes in A) triglyceride, B) NAFLD-liver fat score, C) fasting insulin, and D) HOMA-IR at 12 months after bariatric surgery.

Table 4.5. Bivariate Spearman correlation between Lp(a), markers of lipoprotein-associated oxidative stress, clinical characteristics, lipid profile, and markers of glycaemia and liver function at baseline before surgery.

	Lp(a), mg/dL	***OxPL-apoB, nM***	***IgG MDA-LDL, RLU***	***IgM MDA-LDL, RLU***	***IgG apoB-IC, RLU***	***IgM apoB-IC, RLU***	***OxLDL, ng/mL***
BMI, kg/m^2	-0.129	-0.064	0.113	-0.075	0.193	0.023	-0.206
Total cholesterol, mmol/L	0.258	0.040	**-0.343****	**-0.384****	-0.119	0.281	**0.452****
Triglyceride, mmol/L	-0.132	-0.092	-0.313	-0.120	-0.102	0.089	0.187
HDL-C, mmol/L	0.180	0.076	-0.306	-0.257	-0.324	0.056	**0.441****
LDL-C, mmol/L	0.299	0.089	-0.244	-0.341	-0.009	**0.384****	**0.382****
ApoB, mg/dL	-0.013	0.013	-0.253	-0.205	-0.151	-0.052	0.265
PCSK9, ng/mL	0.223	**0.665****	0.042	0.211	0.290	0.206	-0.110
Fasting glucose, mmol/L	0.174	0.040	**-0.383****	-0.119	-0.214	0.026	**0.543****
Fasting insulin, mU/L	-0.090	-0.109	-0.038	-0.134	0.142	0.066	-0.035
HOMA-IR	-0.075	-0.113	-0.156	-0.132	0.076	0.078	0.191
Triglyceride, mmol/L	-0.140	-0.123	0.199	0.050	0.300	0.176	-0.244
ALT, U/L	0.139	-0.083	-0.147	0.137	-0.215	-0.104	0.136
AST, U/L	**0.343****	-0.088	-0.103	0.161	-0.527	-0.268	**0.539****
GGT, U/L	-0.337	-0.131	-0.125	-0.126	-0.156	-0.100	-0.014
NAFLD-liver fat score	0.055	-0.142	0.149	0.019	0.057	-0.092	-0.133

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; GGT, gamma-glutamyl transferase; HOMA-IR, HDL-C, high-density lipoprotein cholesterol; homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease. ***P*<0.01

Table 4.6. Bivariate Spearman correlation between changes in Lp(a), markers of lipoprotein-associated oxidative stress, clinical characteristics, and lipid profile at 6 months and 12 months after surgery.

	Δ Lp(a), mg/dL	***Δ OxPL-apoB, nM***	***Δ IgG MDA-LDL, RLU***	***Δ IgM MDA-LDL, RLU***	***Δ IgG apoB-IC, RLU***	***Δ IgM apoB-IC, RLU***	***Δ OxLDL, ng/mL***
6 months							
ΔBMI, kg/m²	-0.058	0.043	-0.109	-0.51	0.251	0.161	0.231
ΔTotal cholesterol, mmol/L	0.097	0.337	-0.015	-0.032	-0.090	-0.001	0.101
Δ Triglyceride, mmol/L	-0.170	-0.095	-0.092	-0.235	0.062	-0.101	-0.118
ΔHDL-C, mmol/L	0.019	0.442	0.098	-0.095	-0.119	0.001	0.269
ΔLDL-C, mmol/L	0.205	0.315	0.009	0,067	-0.090	0.015	0.154
ΔApoB, mg/dL	-0.004	0.021	0.049	0.087	-0.161	-0.087	0.131
ΔPCSK9, ng/mL	0.296	**0.561****	-0.073	0.438	-0.113	**0.509****	0.352
12 months							
ΔBMI, kg/m²	-0.151	-0.261	0.227	0.185	0.171	-0.199	-0.064
ΔTotal cholesterol, mmol/L	-0.113	0.086	-0.215	0.145	-0.194	-0.118	**0.456****
Δ Triglyceride, mmol/L	**-0.373****	**-0.349****	0.023	-0.040	-0.095	-0.102	-0.054
ΔHDL-C, mmol/L	0.129	0.207	-0.042	-0.169	0.024	0.130	**0.535****
ΔLDL-C, mmol/L	-0.033	0.161	-0.200	0.221	-0.160	-0.082	**0.402****
ΔApoB, mg/dL	-0.154	0.048	**-0.405****	0.129	**-0.396****	-0.068	**0.581****
ΔPCSK9, ng/mL	0.052	**0.474****	-0.118	0.355	-0.381	0.050	0.173

Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; IC, immune complex; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; PCSK9, proprotein convertase subtilisin/kexin 9; RLU, reactive light units. ***P*<0.01

Table 4.7. Bivariate Spearman correlation between changes in Lp(a) and markers of lipoprotein-associated oxidative stress, glycaemia, and liver function.

	Δ Lp(a), mg/dL	***Δ OxPL-apoB, nM***	***Δ IgG MDA-LDL, RLU***	***Δ IgM MDA-LDL, RLU***	***Δ IgG apoB-IC, RLU***	***Δ IgM apoB-IC, RLU***	***Δ OxLDL, ng/mL***
6 months							
Δ Fasting glucose, mmol/L	-0.205	0.050	-0.109	-0.51	0.251	0.161	0.231
Δ Fasting insulin, mU/L	-0.015	-0.113	-0.197	-0.122	0.094	0.148	-0.223
Δ HOMA-IR	-0.065	-0.096	-0.183	-0.177	-0.009	0.195	-0.117
Δ Triglyceride, mmol/L	-0.170	-0.095	-0.092	-0.235	0.062	-0.101	-0.118
Δ ALT, U/L	-0.011	-0.042	-0.152	0.099	0.331	0.129	-0.182
Δ AST, U/L	-0.004	-0.086	0.168	-0.064	0.222	0.380	0.078
Δ GGT, U/L	-0.121	-0.282	-0.148	-0.084	-0.053	-0.183	0.027
Δ NAFLD-liver fat score	-0.054	-0.203	-0.340	-0.102	0.186	0.123	-0.228
12 months							
Δ Fasting glucose, mmol/L	**-0.372****	0.127	-0.202	-0.023	-0.124	-0.023	0.137
Δ Fasting insulin, mU/L	**-0.339****	-0.215	0.097	0.017	0.066	-0.147	0.081
Δ HOMA-IR	**-0.379****	-0.146	0.012	0.034	-0.019	-0.191	0.090
Δ Triglyceride, mmol/L	**-0.373****	**-0.349****	0.023	-0.040	-0.095	-0.102	-0.054
Δ ALT, U/L	-0.190	-0.241	-0.149	-0.180	0.005	-0.024	-0.247
Δ AST, U/L	-0.254	-0.174	-0.174	-0.241	-0.291	-0.136	-0.015
Δ GGT, U/L	-0.068	-0.244	0.171	-0.185	0.116	0.055	0.058
Δ NAFLD-liver fat score	-0.309	**-0.384****	0.207	0.043	0.195	-0.111	-0.138

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase, GGT, gamma-glutamyl transferase; HOMA-IR, homeostatic model assessment for insulin resistance; IC, immune complex; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; NAFLD, non-alcoholic fatty liver disease; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100. ***P*<0.01

Subgroup analyses in patients with and without type 2 diabetes

The increase in Lp(a) levels after surgery was significant in patients without type 2 diabetes but did not achieve statistical significance in patients with type 2, although the mean absolute changes were similar between the two groups. (Table 4.8). Reductions in OxPL-apoB and OxPL-apoB to Lp(a) ratio were significant in both patients with and without type 2 diabetes. IgG MDA-LDL, IgM MDA-LDL, IgG apoB-IC and IgM apoB-IC all decreased significantly at 12 months after surgery in both patients with and without type 2 diabetes. OxLDL, however, did not change significantly in either group.

Subgroup analyses in patients undergoing Roux-en-Y gastric bypass and sleeve gastrectomy

The increase in Lp(a) was significant in patients who underwent sleeve gastrectomy (Table 4.9). Significant reductions in OxPL-apoB were observed following both RYGB and sleeve gastrectomy. There was a reduction in OxPL-apoB to Lp(a) following RYGB. Both groups had significant decreases in IgG MDA-LDL, IgG apoB-IC and IgM apoB-IC. No significant changes were observed in OxLDL in both groups.

Table 4.8. Comparison of clinical characteristics and changes in lipoprotein(a) and markers of lipoprotein-associated oxidative stress in patients with and without type 2 diabetes.

	Type 2 diabetes (n=37)			No type 2 diabetes (n=22)		
	Baseline	**12 months**	**Absolute change**	**Baseline**	**12 months**	**Absolute change**
Clinical characteristics						
Age, y	51.4 (10.5)			47.9 (8.8)		
Statin, n (%)	26 (70)			4 (18)		
BMI, kg/m^2	48 (44–57)	**35 (31–38)****	-15.4 (5.9)	49 (45–52)	**32 (29–36)****	-15.8 (4.8)
Lipoprotein(a) and markers of lipoprotein-associated oxidative stress						
Lp(a), mg/dL	10.3 (4.3–32.0)	16.1 (5.8–39.0)	5.3 (16.3)	10.1 (3.1–33.1)	**17.7 (4.8–41.5)****	8.7 (10.5)
OxLDL, ng/mL	50.3 (33.7–129.8)	51.9 (32.6–121.2)	-0.3 (-23.3–11.8)	57.3 (39.8–129.8)	**77.8 (35.1–142.8)**	-2.0 (-10.6–19.6)
OxPL-apoB, nM	5.0 (3.4–7.1)	**4.3 (3.1–6.1)***	-1.9 (3.9)	4.7 (3.1–7.9)	**3.6 (2.3–5.1)****	-1.5 (2.4)
OxPL-apoB to Lp(a) ratio	0.55 (0.23–1.01)	**0.27 (0.15–0.78)***	-0.09 (-0.41–0.07)	0.76 (0.17–1.44)	**0.22 (0.14–0.56)****	-0.12 (-0.70–-0.01)
IgG MDA-LDL, RLU	16977 (11860–27046)	**11710 (8922–16059)****	-6432 (12813)	16267 (11068–21433)	**11786 (8637–16014)****	-4086 (5960)
IgM MDA-LDL, RLU	31623 (20641–53362)	**26664 (15679–40471)****	-7373 (16039)	38779 (18212)	**28493 (16575)****	-10635 (12931)
IgG IC, RLU	36248 (19742–44945)	**20353 (13732–26127)****	-18058 (19756)	28733 (20493–44214)	**12430 (9350–21731)****	-16147 (22129)
IgM IC, RLU	20633 (15784–28625)	**16706 (9257–25640)****	-6023 (-11680–337)	18936 (12757–26353)	**11616 (8488–17363)****	-4005 (-12346–-100)

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables. Comparison of variables post-intervention with baseline was performed using paired t-test for parametric and Wilcoxon signed-rank test for non-parametric variables. **P*<0.05, ***P*<0.01

Abbreviations: BMI, body mass index; IC, immune complex; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; PCSK9, proprotein convertase subtilisin/kexin 9; RLU, reactive light units.

Table 4.9. Comparison of clinical characteristics and changes in lipoprotein(a) and markers of lipoprotein-associated oxidative stress in patients who underwent Roux-en-Y gastric bypass and sleeve gastrectomy.

	Roux-en-y gastric bypass (n=31)			Sleeve gastrectomy (n=19)		
	Baseline	**12 months**	**Absolute change**	**Baseline**	**12 months**	**Absolute change**
Clinical characteristics						
Age, y	47.8 (9.4)			52.8 (9.7)		
Type 2 diabetes	22 (71)			8 (42)		
Statin, n (%)	15 (48)			10 (53)		
BMI, kg/m^2	49 (6)	**32 (5)****	-16.5 (5.1)	47 (7)	**34 (6)****	-13.1 (4.8)
Lipoprotein(a) and markers of lipoprotein-associated oxidative stress						
Lp(a), mg/dL	10.3 (4.2–31.8)	18.0 (5.4–36.6)	6.1 (14.8)	10.0 (4.6–28.6)	**10.7 (4.3–42.9)***	5.3 (9.0)
OxLDL, ng/mL	50.3 (34.0–114.2)	76.6 (32.8–118.9)	-7.5 (-16.5–8.8)	56.6 (37.5–135.3)	76.4 (40.5–149.6)	11.6 (-3.3–40.2)
OxPL-apoB, nM	4.8 (3.0–7.1)	**4.0 (3.2–5.2)***	-1.5 (3.6)	4.9 (3.4–7.8)	**3.2 (2.0–5.5)***	-1.7 (3.0)
OxPL-apoB to Lp(a) ratio	0.50 (0.22–1.02)	**0.22 (0.15–0.69)****	-0.41 (0.78)	0.63 (0.25–1.13)	**0.29 (0.15–0.80)**	-0.16 (0.39)
IgG MDA-LDL, RLU	16977 (12484–26043)	**10705 (8308–15386)****	-5366 (5169)	14882 (10983–27287)	**13463 (8806–15957)***	-7368 (15992)
IgM MDA-LDL, RLU	31623 (21653–54558)	**26664 (15631–31934)****	-11242 (17345)	32102 (19878–47179)	**28735 (12929–42494)**	-3190 (10017)
IgG IC, RLU	36442 (27272–40378)	**18046 (10678–21696)****	-21871 (20160)	28718 (18201–43045)	**15173 (9506–22373)****	-14000 (21956)
IgM IC, RLU	19780 (15230–27486)	**13580 (8890–20668)****	-8794 (18083)	21116 (12063–27223)	**14992 (7896–19353)***	-23768 (79266)

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables. Comparison of variables post-intervention with baseline was performed using paired t-test for parametric and Wilcoxon signed-rank test for non-parametric variables. * P<0.05, ** P<0.01

Abbreviations: BMI, body mass index; IC, immune complex; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; PCSK9, proprotein convertase subtilisin/kexin 9; RLU, reactive light units.

4.5 DISCUSSION

This is the first study to simultaneously assess the effect of bariatric surgery on Lp(a), OxPL-apoB and related biomarkers of oxidised lipoproteins in patients with severe obesity. In concert with expected improvements in BMI, insulin resistance measures, and liver function, bariatric surgery resulted in an increase in Lp(a). In contrast, biomarkers of oxidiSed lipoproteins, including OxPL-apoB, IgG and IgM MDA-LDL and apoB-IC, but not OxLDL, showed significant decreases following bariatric surgery. Overall, these data suggest that along with improved liver function and insulin resistance, a global improvement in oxidative stress that is manifested by significant reductions in specific biomarkers of oxidised lipoproteins is observed after bariatric surgery.

The increase in Lp(a) may seem unexpected, particularly as plasma levels of other lipoproteins showed more modest changes, albeit many subjects were on lipid modifying medications. Lp(a) plasma levels are primarily mediated by hepatocyte synthetic activity rather than clearance mechanisms,[39] and it is not surprising that an improvement in liver function may also improve production and secretion of Lp(a) leading to higher plasma levels. Prior studies have shown that in advanced liver disease or non-alcoholic steatohepatitis, plasma levels of Lp(a) are reduced.[40] As Lp(a) is secreted from the liver, one potential explanation for the increase in Lp(a) would be the improvement in hepatic steatosis and liver function following surgery. Secondly, the post-surgical rise in Lp(a) could also be a result of reduced hepatic clearance of apolipoprotein(a) in triglyceride-rich particles as triglyceride levels decreases and this is consistent with the inverse relationship between Lp(a) and triglyceride levels observed in our study, as well as previous cross-sectional studies.[41-43] Thirdly, there is also an established inverse association between Lp(a) and insulin resistance and insulin levels as demonstrated in previous studies[19-21] and it is proposed that this may be a result of insulin-mediated suppression of hepatic apolipoprotein(a) synthesis.[44] These proposed mechanisms for the increase in Lp(a) may be more applicable in patients with type 2 diabetes as similar correlation was not maintained in patients without type 2 diabetes. This can be expected given that even among patients with severe obesity, the presence of type 2 diabetes would

indicate a more severe phenotype of metabolic syndrome and therefore a more marked effect of insulin resistance and hepatic steatosis on Lp(a) synthesis.

It was previously been demonstrated that Lp(a) binds and transports OxPL, and that the OxPL-apoB assay often reflects the pro-inflammatory effects of Lp(a) since it contributes a large proportion of the OxPL measured with this assay,[7, 10, 37, 45] hence a reduction in OxPL-apoB following surgery may also seem unexpected. However, although Lp(a) itself can become oxidised and autogenerate OxPL, most OxPL are generated elsewhere, such as in the liver, and likely transferred to and carried by Lp(a). Therefore, synthesis and secretion of Lp(a) and generation of OxPL are not necessarily conjoined pathophysiologically in all instances. In the case of the physiological milieu following bariatric surgery, it can be anticipated that with the increased synthetic capacity of the liver, the overall oxidative stress the hepatocytes are exposed to, and therefore the amount of OxPL generated, are markedly reduced, that likely explains the decline in OxPL-apoB. Consistent with this, the OxPL-apoB to Lp(a) ratio was also significantly reduced. OxPL have been shown in previous studies to accumulate in non-alcoholic steatohepatitis, and are involved in the pathogenesis of hepatic steatosis.[46, 47]

The experience of therapeutic interventions evaluating changes in OxPL-apoB is not as well established as the database for the baseline levels predicting future risk of myocardial infarction,[37, 48] stroke,[49] peripheral arterial disease[31] and aortic stenosis.[50, 51] However, it has been noted that OxPL-apoB can be reduced up to 89% by antisense oligonucleotides to apo(a)[52] and ~20% by niacin,[53] and although there is significant individual variability, can be increased ~20% by statins in some patients,[54] all of which also affect its main lipoprotein carrier, Lp(a), in the same direction. However, the observation of a divergence of Lp(a) and OxPL-apoB levels following bariatric surgery is reported for the first time, and does suggest a unique pathophysiological relationship between the metabolism of Lp(a) and the generation and transport of OxPL following bariatric surgery.

We have also found significant reductions in autoantibodies to MDA-LDL and apoB-ICs following bariatric surgery which are also independent of surgical procedure and presence of diabetes. Although previous studies have suggested the that IgM autoantibodies and apoB-ICs may be protective and have potential atherosclerosis-

modulating effects in steady state,[5] their levels are increased in the acute phase and states of inflammation[8]. Their reduction following bariatric surgery, along with IgG autoantibodies and apoB-IC are therefore likely to reflect the state of heightened inflammation in severe obesity which then improves following surgery.

In contrast with overall reductions in markers of oxidative stress, levels of OxLDL did not change significantly after bariatric surgery. Comparison of OxLDL data with OxPL-apoB have also only been carried out in one prior study.[55] The assay used to measure OxLDL is based on murine monoclonal antibody 4E6, whose binding to OxLDL has been shown to be completed by unoxidised LDL (likely an apoB epitope) but at higher concentrations, suggesting it detects both OxLDL and unoxidised LDL.[56] Furthermore, spiking samples of known OxLDL levels with unoxidised LDL results in increases in OxLDL, which should not occur if the antibody was specific to OxLDL.[57] These data suggest that OxLDL as measured by the Mercodia assay used in this study does not reflect a true measure of OxLDL. This can also be appreciated by the fact that the medical weight management group, which has a lower BMI but has significantly higher LDL, also has higher OxLDL levels.

Given that it is impossible to create a true control group for a human study into the effects of bariatric surgery, we have undertaken a pre-and post-intervention study with the inclusion of a parallel group of patients who underwent medical weight management. Due to the study being observational in design, we had no control over the choice of intervention for patients hence the difficulty in matching. Younger patients with higher BMIs and less comorbidities are more likely to be offered surgical intervention with less delay, and hence the difference in age and BMI. We acknowledge that a difference in age and presence of diabetes may be a confounding factor when contrasting between surgical and non-surgical groups. However, it is also important to note that the glycaemic control was favourable and similar in both groups. In addition to our primary aim of assessing the changes in Lp(a) and OxPL-apoB after bariatric surgery, we have also conducted multiple analyses into correlations between variations and subgroup comparisons simultaneously, raising a potential issue surrounding multiple testing. We acknowledge that these are exploratory in nature but does provide observations of interest that may help provide some mechanistic explanation to the bariatric surgery-

related changes described. Lastly, whilst we were able to demonstrate significant changes in our primary outcome measures, comparison of variables in subgroup analyses is limited by the sample size of our cohort.

In conclusion, bariatric surgery results in additional beneficial functions reflected by improved measures of oxidised lipoproteins. These biomarkers can be used to further understand the pathophysiological effects of bariatric surgery. Whether changes in these biomarkers will reflect long-term benefits and hard endpoints in patients with obesity awaits to be determined.

4.6 AUTHOR CONTRIBUTIONS

Study concept and design were by J.H. Ho and H. Soran. J.H. Ho, S. Adam, S. Azmi, S. Dhage undertook patient recruitment and assessments. J.H. Ho, Y. Liu, and X. Yang performed laboratory analyses. J.H. Ho performed the data analyses and undertook interpretation of findings with S. Tsimikas and H. Soran. J.H. Ho produced the first draft and the final version with S. Tsimikas and H. Soran. S. Adam, A. Syed, B.J. Ammori, R. Donn, A. Heald, J.M. Gibson, R.A. Malik, and P.N. Durrington provided critical review for important intellectual content.

4.7 ACKNOWLEDGEMENTS

This study was supported by a non-restricted research donation from AMGEN and the Lipid Disease Fund. We acknowledge support from the National Institute of Health Research/Wellcome Trust Clinical Research Facility (Manchester, UK).

4.8 DECLARATION OF INTERESTS

S. Tsimikas is a co-inventor and receives royalties from patents owned by UCSD on oxidation-specific antibodies and of biomarkers related to oxidised lipoproteins and is a co-founder and has an equity interest in Oxitope, Inc and its affiliates (“Oxitope”) as well as in Kleanthi Diagnostics, LLC (“Kleanthi”). The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

The remaining authors have no conflict of interests to declare.

4.9 REFERENCES

1. Furukawa S, Fujita T, Shimabukuro M, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest.* 2004;114:1752-1761.

2. Fernandez-Sanchez A, Madrigal-Santillan E, Bautista M, et al. Inflammation, oxidative stress, and obesity. *Int J Mol Sci.* 2011;12:3117-3132.

3. Glass CK, Witztum JL. Atherosclerosis. the road ahead. *Cell.* 2001;104:503-516.

4. Taleb A, Witztum JL, Tsimikas S. Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark Med.* 2011;5:673-694.

5. Ravandi A, Boekholdt SM, Mallat Z, et al. Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study. *J Lipid Res.* 2011;52:1829-1836.

6. Berliner JA, Leitinger N, Tsimikas S. The role of oxidized phospholipids in atherosclerosis. *J Lipid Res.* 2009;50 Suppl:S207-212.

7. Tsimikas S, Brilakis ES, Miller ER, et al. Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. *N Engl J Med.* 2005;353:46-57.

8. Tsimikas S, Bergmark C, Beyer RW, et al. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J Am Coll Cardiol.* 2003;41:360-370.

9. Tsimikas S, Lau HK, Han KR, et al. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation.* 2004;109:3164-3170.

10. Leibundgut G, Scipione C, Yin H, et al. Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a). *J Lipid Res.* 2013;54:2815-2830.

11. Tsimikas S, Clopton P, Brilakis ES, et al. Relationship of oxidized phospholipids on apolipoprotein B-100 particles to race/ethnicity, apolipoprotein(a) isoform size, and cardiovascular risk factors: results from the Dallas Heart Study. *Circulation.* 2009;119:1711-1719.

12. Bennet A, Di Angelantonio E, Erqou S, et al. Lipoprotein(a) levels and risk of future coronary heart disease: large-scale prospective data. *Arch Intern Med.* 2008;168:598-608.

13. Emerging Risk Factors C, Erqou S, Kaptoge S, et al. Lipoprotein(a) concentration and the risk of coronary heart disease, stroke, and nonvascular mortality. *JAMA.* 2009;302:412-423.

14. Waldeyer C, Makarova N, Zeller T, et al. Lipoprotein(a) and the risk of cardiovascular disease in the European population: results from the BiomarCaRE consortium. *Eur Heart J.* 2017;38:2490-2498.

15. Nordestgaard BG, Langsted A. Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology. *J Lipid Res.* 2016;57:1953-1975.

16. Durrington PN, Schofield JD, Siahmansur T, Soran H. Lipoprotein (a): gene genie. *Curr Opin Lipidol.* 2014;25:289-296.

17. Capoulade R, Chan KL, Yeang C, et al. Oxidized Phospholipids, Lipoprotein(a), and Progression of Calcific Aortic Valve Stenosis. *J Am Coll Cardiol.* 2015;66:1236-1246.

18. Tsimikas S, Duff GW, Berger PB, et al. Pro-inflammatory interleukin-1 genotypes potentiate the risk of coronary artery disease and cardiovascular events mediated by oxidized phospholipids and lipoprotein(a). *J Am Coll Cardiol.* 2014;63:1724-1734.

19. Marzano L, Colussi G, Del Torre M, Sechi LA, Catena C. Relationships of plasma lipoprotein(a) levels with insulin resistance in hypertensive patients. *Metabolism.* 2014;63:1439-1446.

20. Rainwater DL, Haffner SM. Insulin and 2-hour glucose levels are inversely related to Lp(a) concentrations controlled for LPA genotype. *Arterioscler Thromb Vasc Biol.* 1998;18:1335-1341.

21. Duell PB, Hagemenas F, Connor WE. The relationship between serum lipoprotein(a) and insulinemia in healthy nondiabetic adult men. *Diabetes Care.* 1994;17:1135-1140.

22. Berk KA, Yahya R, Verhoeven AJM, et al. Effect of diet-induced weight loss on lipoprotein(a) levels in obese individuals with and without type 2 diabetes. *Diabetologia.* 2017;60:989-997.

23. Faghihnia N, Tsimikas S, Miller ER, Witztum JL, Krauss RM. Changes in lipoprotein(a), oxidized phospholipids, and LDL subclasses with a low-fat high-carbohydrate diet. *J Lipid Res.* 2010;51:3324-3330.

24. Silaste ML, Rantala M, Alfthan G, et al. Changes in dietary fat intake alter plasma levels of oxidized low-density lipoprotein and lipoprotein(a). *Arterioscler Thromb Vasc Biol.* 2004;24:498-503.

25. Yadav R, Hama S, Liu Y, et al. Effect of Roux-en-Y Bariatric Surgery on Lipoproteins, Insulin Resistance, and Systemic and Vascular Inflammation in Obesity and Diabetes. *Front Immunol.* 2017;8:1512.

26. Adam S, Liu Y, Siahmansur T, et al. Bariatric surgery as a model to explore the basis and consequences of the Reaven hypothesis: Small, dense low-density lipoprotein and interleukin-6. *Diab Vasc Dis Res.* 2019;16:144-152.

27. Schauer PR, Bhatt DL, Kirwan JP, et al. Bariatric surgery versus intensive medical therapy for diabetes--3-year outcomes. *N Engl J Med.* 2014;370:2002-2013.

28. Sjostrom L, Peltonen M, Jacobson P, et al. Bariatric surgery and long-term cardiovascular events. *JAMA.* 2012;307:56-65.

29. Vest AR, Heneghan HM, Agarwal S, Schauer PR, Young JB. Bariatric surgery and cardiovascular outcomes: a systematic review. *Heart.* 2012;98:1763-1777.

30. Moussa O, Ardissino M, Heaton T, et al. Effect of bariatric surgery on long-term cardiovascular outcomes: a nationwide nested cohort study. *Eur Heart J.* 2020.

31. Bertoia ML, Pai JK, Lee JH, et al. Oxidation-specific biomarkers and risk of peripheral artery disease. *J Am Coll Cardiol.* 2013;61:2169-2179.

32. Viney NJ, Yeang C, Yang X, Xia S, Witztum JL, Tsimikas S. Relationship between "LDL-C", estimated true LDL-C, apolipoprotein B-100, and PCSK9 levels following lipoprotein(a) lowering with an antisense oligonucleotide. *J Clin Lipidol.* 2018;12:702-710.

33. Kotronen A, Peltonen M, Hakkarainen A, et al. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology.* 2009;137:865-872.

34. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* 1985;28:412-419.

35. Leibundgut G, Arai K, Orsoni A, et al. Oxidized phospholipids are present on plasminogen, affect fibrinolysis, and increase following acute myocardial infarction. *J Am Coll Cardiol.* 2012;59:1426-1437.

36. Arai K, Orsoni A, Mallat Z, et al. Acute impact of apheresis on oxidized phospholipids in patients with familial hypercholesterolemia. *J Lipid Res.* 2012;53:1670-1678.

37. Tsimikas S, Willeit P, Willeit J, et al. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J Am Coll Cardiol.* 2012;60:2218-2229.

38. Tsimikas S, Witztum JL, Miller ER, et al. High-dose atorvastatin reduces total plasma levels of oxidized phospholipids and immune complexes present on apolipoprotein B-100 in patients with acute coronary syndromes in the MIRACL trial. *Circulation.* 2004;110:1406-1412.

39. Schmidt K, Noureen A, Kronenberg F, Utermann G. Structure, function, and genetics of lipoprotein (a). *J Lipid Res.* 2016;57:1339-1359.

40. Konishi K, Miyake T, Furukawa S, et al. Advanced fibrosis of non-alcoholic steatohepatitis affects the significance of lipoprotein(a) as a cardiovascular risk factor. *Atherosclerosis.* 2020;299:32-37.

41. Bartens W, Rader DJ, Talley G, Brewer HB, Jr. Decreased plasma levels of lipoprotein(a) in patients with hypertriglyceridemia. *Atherosclerosis.* 1994;108:149-147.

42. Werba JP, Safa O, Gianfranceschi G, Michelagnoli S, Sirtori CR, Franceschini G. Plasma triglycerides and lipoprotein(a): inverse relationship in a hyperlipidemic Italian population. *Atherosclerosis.* 1993;101:203-211.

43. Hernandez C, Chacon P, Garcia-Pascual L, Simo R. Differential influence of LDL cholesterol and triglycerides on lipoprotein(a) concentrations in diabetic patients. *Diabetes Care.* 2001;24:350-355.

44. Neele DM, de Wit EC, Princen HM. Insulin suppresses apolipoprotein(a) synthesis by primary cultures of cynomolgus monkey hepatocytes. *Diabetologia.* 1999;42:41-44.

45. Bergmark C, Dewan A, Orsoni A, et al. A novel function of lipoprotein [a] as a preferential carrier of oxidized phospholipids in human plasma. *J Lipid Res.* 2008;49:2230-2239.

46. Sun X, Seidman JS, Zhao P, et al. Neutralization of Oxidized Phospholipids Ameliorates Non-alcoholic Steatohepatitis. *Cell Metab.* 2020;31:189-206 e188.

47. Que X, Hung MY, Yeang C, et al. Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice. *Nature.* 2018;558:301-306.

48. Byun YS, Lee JH, Arsenault BJ, et al. Relationship of oxidized phospholipids on apolipoprotein B-100 to cardiovascular outcomes in patients treated with intensive versus moderate atorvastatin therapy: the TNT trial. *J Am Coll Cardiol.* 2015;65:1286-1295.

49. Byun YS, Yang X, Bao W, et al. Oxidized Phospholipids on Apolipoprotein B-100 and Recurrent Ischemic Events Following Stroke or Transient Ischemic Attack. *J Am Coll Cardiol.* 2017;69:147-158.

50. Kamstrup PR, Hung MY, Witztum JL, Tsimikas S, Nordestgaard BG. Oxidized Phospholipids and Risk of Calcific Aortic Valve Disease: The Copenhagen General Population Study. *Arterioscler Thromb Vasc Biol.* 2017;37:1570-1578.

51. Zheng KH, Tsimikas S, Pawade T, et al. Lipoprotein(a) and Oxidized Phospholipids Promote Valve Calcification in Patients With Aortic Stenosis. *J Am Coll Cardiol.* 2019;73:2150-2162.

52. Tsimikas S, Karwatowska-Prokopczuk E, Gouni-Berthold I, et al. Lipoprotein(a) Reduction in Persons with Cardiovascular Disease. *N Engl J Med.* 2020;382:244-255.

53. Yeang C, Hung MY, Byun YS, et al. Effect of therapeutic interventions on oxidized phospholipids on apolipoprotein B100 and lipoprotein(a). *J Clin Lipidol.* 2016;10:594-603.

54. Tsimikas S, Gordts P, Nora C, Yeang C, Witztum JL. Statin therapy increases lipoprotein(a) levels. *Eur Heart J.* 2019.

55. Ky B, Burke A, Tsimikas S, et al. The influence of pravastatin and atorvastatin on markers of oxidative stress in hypercholesterolemic humans. *J Am Coll Cardiol.* 2008;51:1653-1662.

56. Holvoet P, Donck J, Landeloos M, et al. Correlation between oxidized low density lipoproteins and von Willebrand factor in chronic renal failure. *Thromb Haemost.* 1996;76:663-669.

57. Tsouli SG, Kiortsis DN, Lourida ES, et al. Autoantibody titers against OxLDL are correlated with Achilles tendon thickness in patients with familial hypercholesterolemia. *J Lipid Res.* 2006;47:2208-2214.

CHAPTER 5: HIGH-DENSITY LIPOPROTEIN-ASSOCIATED MIRNA IS INCREASED FOLLOWING ROUX-EN-Y GASTRIC BYPASS SURGERY FOR SEVERE OBESITY

Published in Journal of Lipid Research

Author's contribution:

The author undertook patient recruitment, study visit assessments, and processing of blood samples for storage. The author also undertook laboratory analyses under supervision apart from the HDL-miRNA assay which was undertaken at the University of New South Wales Sydney by Dr Kwok Leung Ong. The author performed data analysis with guidance from Dr Fatiha Tabet. He undertook research of available literature, wrote the first draft, and produced the final manuscript with Dr Fatiha Tabet and Dr Handrean Soran.

High-density lipoprotein-associated miRNA is increased following Roux-en-Y gastric bypass surgery for severe obesity

Jan Hoong Ho[1,2], Kwok Leung Ong[3], Luisa F. Cuesta Torres[3], Yifen Liu[1], Safwaan Adam[1,2], Zohaib Iqbal[1,2], Shaishav Dhage[1,2], Basil J Ammori[4], Akheel A Syed[5], Kerry-Anne Rye[3], Fatiha Tabet[3*], Handrean Soran[1,2*]

[1]Lipid Research Group, Division of Cardiovascular Sciences, University of Manchester, Manchester, UK

[2]Cardiovascular Trials Unit, Manchester University NHS Foundation Trust, Manchester, UK

[3]Lipid Research Group, School of Medical Sciences, UNSW Sydney, Sydney, Australia

[4]Department of Surgery, Salford Royal NHS Foundation Trust, Salford, UK

[5]Department of Endocrinology, Salford Royal NHS Foundation Trust, Salford, UK

*Contributed equally

Corresponding author:

Dr Handrean Soran MSc, MD, FRCP

Consultant Physician and Endocrinologist

E-mail: hsoran@aol.com; handrean.soran@mft.nhs.uk

5.1 ABSTRACT

Aim: We have recently demonstrated that high-density lipoproteins (HDL) transport microRNAs (miRNAs) in plasma. Here we aim to investigate how HDL-associated miRNAs are regulated in severe obesity and more importantly, how weight loss after Roux-en-Y gastric bypass surgery (RYGB) affects HDL-miRNAs.

Methods: Plasma HDL were isolated from patients with severe obesity (n=53) before, 6 and 12 months after RYGB by immunoprecipitation using goat anti-human apoA-I microbeads. HDL were also isolated from 18 healthy participants. miRNAs were extracted from isolated HDL and levels of miR-24, miR-126, miR-222, miR-223 were determined by TaqMan miRNA assays.

Results: HDL-associated miR-126, miR-222 and miR-223 levels, but not miR-24 levels, were significantly higher in patients with severe obesity compared to healthy controls. There were significant increases in HDL-associated miR-24, miR-222 and miR-223 levels at 12 months after RYGB. Cholesterol efflux capacity and paraoxonase (PON1) activity were increased and intracellular adhesion molecule-1 (ICAM-1) levels decreased at 12 months after RYGB. The increases in HDL-associated miR-24 and miR-223 were positively correlated with increase in cholesterol efflux capacity (r=0.326, P=0.027 and r=0.349, P=0.017 respectively). An inverse correlation was observed between HDL-associated miR-223 and ICAM-1 at baseline.

Conclusions: HDL-associated miRNAs are differentially regulated in healthy versus patients with severe obesity. The increase in HDL-associated miRNAs following RYGB may reflect enhancement of HDL function and may contribute to the metabolic improvements observed. Understanding how miRNAs are regulated in obesity before and after weight reduction interventions has the potential to identify novel treatment strategies for obesity and related metabolic disorders.

KEYWORDS

Obesity, HDL miRNA, weight loss, bariatric surgery, Roux-en-y gastric bypass

5.2 INTRODUCTION

The global prevalence of obesity has doubled over the last three to four decades and continues to rise progressively.[1, 2] Bariatric surgery results in durable reduction in weight and sustained improvement in metabolic and cardiovascular outcomes, both in a weight-dependent and weight-independent manner.[3, 4] The precise molecular mechanisms driving the metabolic effects of bariatric surgery remain to be fully established.

Micro-ribonucleic acids (miRNAs) are small non-coding RNAs that negatively regulate messenger RNAs (mRNAs) through target transcript degradation or by inhibiting translation[5] MiRNAs are involved in a diverse range of biological pathways and have been implicated in the biological processes underlying obesity and the associated cardiometabolic disease[5-9] Altered miRNA expression, including increased miR-24[10] and decreased miR-126 in adipose tissue,[11] and increased miR-222[7] and decreased miR-223[12] in the circulation have all been described previously in obesity. MiRNAs act at the intracellular level and are transported between cells in association with lipid-based vesicles, lipoproteins, and lipid-free protein complexes.[13] We and others have recently demonstrated the involvement of high-density lipoproteins (HDL) in the transport of functional miRNAs within an intercellular communication network, with delivery of a specific miRNA (miR-223) to endothelial cells, contributing to the anti-inflammatory capacity of HDL.[13-15]

HDL cholesterol (HDL-C) levels have been shown to correlate inversely with cardiovascular disease risk[16] and a clear link exists between excess weight and adiposity in obesity and low HDL-C levels.[17] Improvements in HDL structure and function have been reported following metabolic surgery,[18] although evidence for its effect on cholesterol efflux capacity have been inconsistent so far.[19-21] Bariatric surgery has been shown to impact on the circulating miRNA signature of obesity[7] and significant changes have been described even prior to significant weight loss.[22] The effect of bariatric surgery on HDL-associated miRNAs has not been investigated and may contribute mechanistically to improved HDL function following bariatric surgery. In this study, we assessed the changes in HDL-associated miR-24, miR-126, miR-222, and miR-223 levels following bariatric surgery.

5.3 MATERIALS AND METHODS

Participants

We recruited 53 patients with severe obesity (BMI 45.6-57.5 kg/m^2 and weight circumference 142±17 cm) who underwent Roux-en-Y gastric bypass surgery (RYGB) at the Salford Royal NHS Foundation Trust tertiary weight management centre (Salford, UK). Patients with acute coronary syndrome within the past 6 months, history of malignancy, anaemia, active infections, HIV, and autoimmune diseases were excluded. Assessments were undertaken at baseline, 6 months, and 12 months after surgery. 18 healthy participants without a history of type 2 diabetes or statin therapy were recruited for comparison. This study was approved by the Greater Manchester Central Research and Ethics Committee. Written informed consent was obtained from all patients prior to participation and study assessments were conducted in accordance with the 1964 Helsinki declaration.

Laboratory analyses

Venous blood samples were obtained from patients between 0900 and 1100 h following an overnight fast of at least 12 h. Glycosylated haemoglobin (HbA1c) was measured using standard laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust (Manchester, UK) on the day of collection. Isolated serum and plasma samples were stored at -80°C until use. Other laboratory measurements were performed at the end of the study.

Total cholesterol and triglyceride were measured using CHOP-PAP and GPO-PAP methods, respectively. Apolipoprotein A-I (apoA-I) and apolipoprotein B-100 (apoB) were measured using immunoturbidimetry. HDL-C was assayed using a second-generation homogenous direct method.[23] Serum paraoxonase (PON1) activity was measured using paraoxon (O,O-diethyl O-(4-nitrophenyl) phosphate) as a substrate (Sigma-Aldrich, St Louis, USA).[24] All these tests were performed on a Randox Daytona+ analyser (Randox Laboratories, Crumlin, UK). The laboratory participated in the RIQAS scheme (Randox International Quality Assessment Scheme; Randox

Laboratories, Dublin, Ireland). Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formula. No patients had triglyceride levels above 4.5 mmol/l.

Adiponectin, leptin, resistin, and intercellular adhesion molecule-1 (ICAM-1) were measured using DuoSet ELISA development kits (R&D Systems, Abingdon, UK), and insulin and glucose using Mercodia ELISA kits (Diagenics Ltd, Milton Keynes, UK). Homeostatic model assessment was used for assessment of insulin resistance (HOMA-IR).[25]

Cholesterol efflux capacity of HDL was determined using a previously validated method.[26-28] The intra- and inter-assay coefficients of variation were 3.9% and 7.3% respectively. Briefly, J774A.1 cells were incubated with 0.2 µCi of radiolabelled ^{3}H-cholesterol in RPMI 1640 medium with 0.2% BSA at 37 °C in a 5% carbon dioxide humidified atmosphere. ABCA1 expression was upregulated using 0.3 mM C-AMP (8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium salt) for 4 hours and the cells incubated with 2.8% (v/v) apoB-depleted serum using polyethylene glycol (PEG MW8000) for 4 hours. The cell media was then collected and the cells were dissolved in 0.5 ml 0.2 N NaOH to determine radioactivity. Cholesterol efflux was expressed as the percentage of radioactivity in the medium from the radioactivity in the cells and medium collectively:

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

HDL isolation

Isolation of HDL was performed by immunoprecipitation of serum (600 µl) as previously described.[29] Serum was applied to a column containing goat anti-human apoA-I antibody covalently coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Academy Bio-Medical Company, Inc. Houston USA). The column was then washed 10 times with Tris-buffered saline (TBS) to remove proteins non-specifically bound to the beads. HDL was then eluted using stripping buffer (0.1 M

acetic acid) and immediately neutralised with 1 M Tris, pH 11 (final concentration, 0.11 M). An Amicon Ultra-15 centrifuge filter unit and an Amicon Ultra-0.5, ultracel-10 membrane were used for further concentration of the samples.

HDL-associated miRNAs

HDL-miRNA levels were assessed using real-time PCR TaqMan miRNA assays as previously described [29]. Total RNA was isolated from HDL using Qiazol miRNAeasy kits (Qiagen, Hilden, Germany) and total RNA was quantified by spectrophotometry. Total RNA was purified and reversed transcribed using the TaqMan microRNA reverse transcription kit (Applied Biosystems) and 7.5 μl of the reverse transcription product was used for detecting specific miRNAs using TaqMan miRNA assay kits (Applied Biosystems). Values were normalised to both *Caenorhabditis elegans* (Cel) miR-39 (which was spiked into the samples after the Qiazol step) and HDL total protein concentration determined by BCA assay (Thermo Scientific, USA). Results were expressed as $2^{-(Ct(microRNA)-Ct(Cel\text{-}miR\text{-}39))}$.

Statistical Analyses

Data analysis was performed using SPSS 24 (IBM, Armonk, NY). Statistical significance was set at two-tailed $P<0.05$. Data were examined for normality based on skewness and kurtosis, and Shapiro-Wilk's W test before analysis. Non-normally distributed variables were normalised prior to analysis using nature logarithmic (ln) transformation. To evaluate the effect of time on clinical characteristics and HDL-associated miRNA levels, one-way univariate repeated measures ANOVA was performed with time (baseline before surgery, and 6 and 12 months after surgery) as the within subject factor. Participants with missing data were excluded from the analysis. As there are a substantial number of participants with missing clinical characteristics at 6 months after surgery, clinical characteristics at baseline were compared to those at 6 and 12 months after surgery using individual paired t-test. Multiple testing correction was performed by Bonferroni correction for the two time-

points, in which the threshold of p value for significance was <0.025. As the miRNA levels were highly correlated with each other, multiple testing corrections for the four miRNAs was not performed. Correlations between different HDL-associated miRNAs, as well as between changes in individual miRNAs and changes in other variables were performed using bivariate Spearman correlation coefficients.

5.4 RESULTS

Study sample and HDL-associated miRNA levels at baseline before surgery

A total of 53 patients with severe obesity and comparison group of 18 healthy participants were included in this study. The clinical characteristics for both groups are summarised in Table 5.1. 29 patients had type 2 diabetes and 26 were statin-treated which remained unchanged following surgery. Blood samples were available for HDL-associated miRNA measurements at baseline and 12 months after surgery for all patients, and at 6 months for 42 patients. There was no significant difference in age, sex, and BMI between those with and without samples at 6 months after surgery (*P*=0.104, 0.305, and 0.949, respectively). As shown in Figure 5.1, HDL-associated miR-126, miR-222 and miR-223 levels, but not HDL-associated miR-24 levels, were significantly elevated in patients with severe obesity compared to healthy participants. The elevation of HDL-associated miR-126, miR-222 and miR-223 levels remained significant after adjusting for age and sex in multivariable linear regression analysis (*P*=0.006, 0.034 and 0.037, respectively).

Table 5.1. Baseline characteristics of patients who underwent surgery and healthy participants.

	RYGB (n=53)	**Healthy (n=18)**
Age, y	48.9 (8.7)	43.3 (11.9)
Female, n (%)	40 (75)	14 (78)
BMI, kg/m^2	49.4 (45.8–57.4)	22.0 (20.5–23.6)
Waist circumference, cm	137.3 (128.5–150.5)	81.0 (71.1–94.0)
SBP, mmHg	131 (120–146)	127 (115–140)
DBP, mmHg	75.4 (13.8)	73.0 (12.5)
Total cholesterol, mmol/L	4.26 (3.79–5.28)	5.26 (4.77–5.87
Triglycerides, mmol/L	1.46 (1.12–1.93)	0.81 (0.70–1.41)
HDL-C, mmol/L	1.03 (0.87–1.32)	1.66 (1.40–1.88)
LDL-C, mmol/L	2.44 (1.96–3.21)	3.29 (2.61–3.60)
ApoA-I, g/L	1.26 (1.15–1.40)	1.55 (1.39–1.84)
ApoB, g/L	0.80 (0.68–1.02)	0.85 (0.77–0.95)

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables.

Abbreviations: ApoA-I, apolipoprotein A-I; apoB, apolipoprotein B-100; BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C low-density lipoprotein cholesterol; SBP, systolic blood pressure.

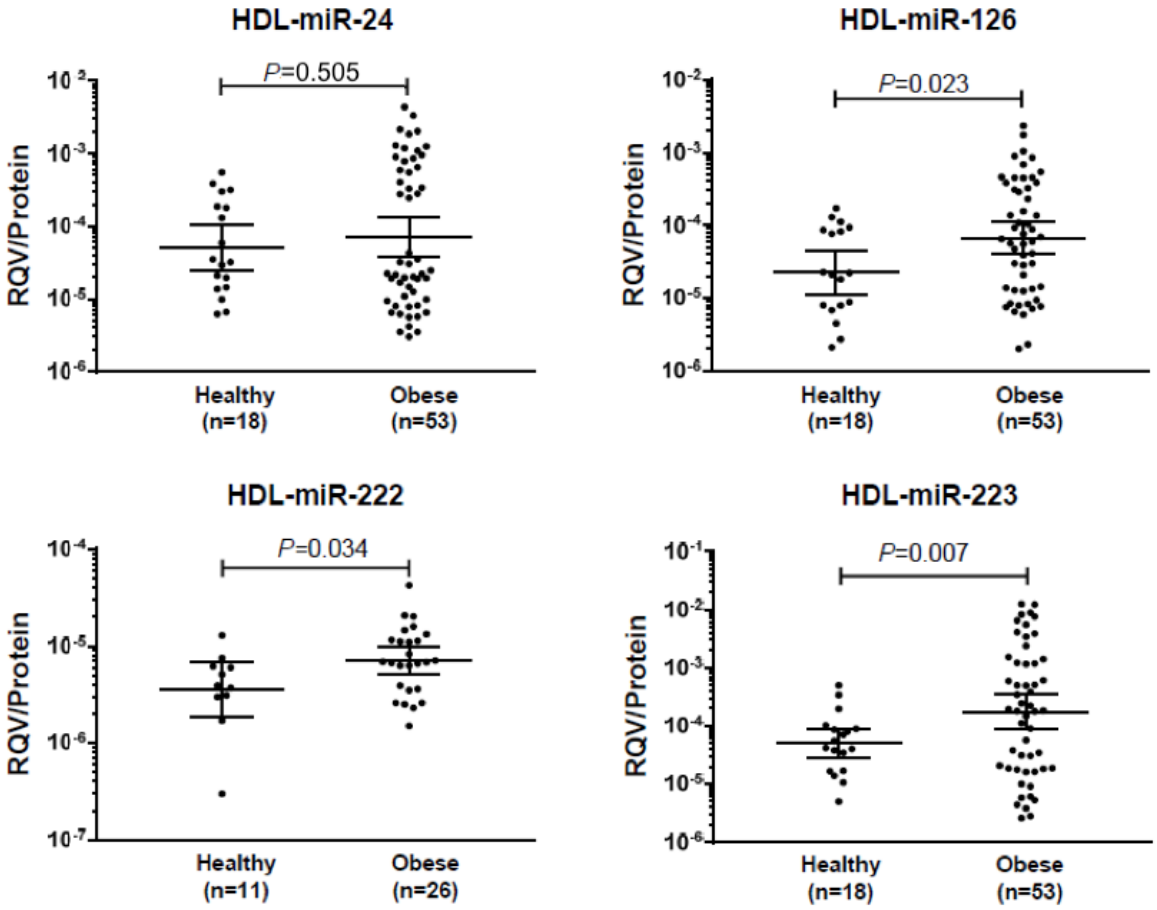


Figure 5.1. Distribution of different HDL-associated miRNA levels in healthy and obese subjects.

Data shown are geometric mean and 95% confidence interval with log scale on the y-axis. Data were compared using independent t-test after log transformation.

Among the patients with severe obesity, all HDL-associated miRNAs showed strong positive correlation with each other at baseline (r=0.461–0.878, all P<0.05). Similar results were found at 6 and 12 months after surgery, although the correlation of HDL-associated miR-222 with miR-126 and miR-223 was attenuated to non-significance at 12 months after surgery (Table 5.2). HDL-associated miR-24, miR-126, and miR-223 levels correlated positively with total cholesterol, HDL-C, LDL-C, and apoB levels (r=0.318 to 0.549, all P<0.05) (Table 5.3). Positive correlations were also observed with cholesterol efflux capacity (miR-24, miR-126 and miR-223; r=0.307 to 0.449; all P<0.05) and PON1 activity (miR-24 and miR-126), r=0.340 and 0.427 respectively, both P<0.05) (Table 5.3). Both HDL-associated miR-126 and miR-223 correlated inversely with ICAM-1 (r=-0.382 and -0.281 respectively, both

P<0.05) (Table 5.3). HDL-associated miR-126, miR-222, and miR-223 levels inversely correlated with diastolic BP (*r*= -0.272 to -0.467, all *P*<0.05) (Table 5.3) and HDL-associated miR-223 inversely correlated with resistin (*r*=-0.275, *P*=0.048) (Table 5.3).

Table 5.2. Cross-sectional bivariate Spearman correlation among different HDL-associated miRNAs at baseline, and 6 and 12 months after surgery.

	miR-24	***miR-126***	***miR-222***	***miR-223***
Before surgery				
miR-24	-			
miR-126	**0.824‡**	-		
miR-222	**0.610‡**	**0.837‡**	-	
miR-223	**0.762‡**	**0.878‡**	**0.461***	-
6 months				
miR-24	-			
miR-126	**0.875‡**	-		
miR-222	**0.709†**	0.387	-	
miR-223	**0.824‡**	**0.859‡**	**0.700†**	-
12 months				
miR-24	-			
miR-126	**0.800‡**	-		
miR-222	**0.646‡**	0.354	-	
miR-223	**0.810‡**	**0.817‡**	0.363	-

*P<0.05, †P<0.01 and ‡P<0.001.

Table 5.3. Spearman correlation between different HDL-associated miRNAs and clinical characteristics at baseline before surgery.

	miR-24	***miR-126***	***miR-222***	***miR-223***
Age, y	-0.012	-0.127	-0.232	0.005
Height, m	-0.019	-0.258	-0.074	-0.042
BMI, kg/m^2	-0.164	-0.122	-0.038	-0.241
Waist circumference, cm	-0.259	-0.108	0.030	-0.256
SBP, mmHg	-0.115	-0.118	-0.124	-0.099
DBP, mmHg	-0.222	**-0.272***	**-0.467***	**-0.320***
Total cholesterol, mmol/L	**0.426†**	**0.549‡**	0.220	**0.461‡**
Triglycerides, mmol/L	0.156	0.071	0.157	0.060
HDL-C, mmol/L	**0.373†**	**0.459‡**	-0.252	**0.495‡**
LDL-C, mmol/L	**0.318***	**0.462‡**	0.288	**0.350***
ApoA-I, g/L	0.127	0.142	-0.218	0.235
ApoB, g/L	**0.372†**	**0.498‡**	0.077	**0.397†**
Cholesterol efflux capacity, %	**0.352***	**0.449†**	0.202	**0.307***
PON1 activity, nmol/ml/min	**0.340***	**0.427†**	-0.069	0.242
HbA1c, mmol/L	-0.021	-0.079	-0.238	-0.085
Fasting glucose, mmol/L	0.025	0.056	-0.213	0.113
Fasting insulin, mU/L	0.087	0.142	-0.201	0.070
HOMA-IR	0.061	0.121	-0.336	0.094
HOMA-B	0.091	0.072	-0.043	-0.023
ICAM-1, ng/mL	-0.111	**-0.382****	-0.127	**-0.281***
Adiponectin, mg/L	0.200	0.095	0.034	0.155
Leptin, ng/mL	0.110	0.055	-0.021	0.002
Resistin, ng/mL	-0.041	-0.090	0.388	**-0.275***

Abbreviations: ApoA-I, apolipoprotein A-I; apoB, apolipoprotein B-100; BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycosylated haemoglobin; HDL-C, high-density lipoprotein cholesterol; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment for insulin resistance; ICAM-1, intercellular adhesion molecule-1; LDL-C low-density lipoprotein cholesterol; PON1, paraoxonase-1; SBP, systolic blood pressure.

*P<0.05, †P<0.01 and ‡P<0.001.

Change in clinical characteristics after surgery

Table 5.4 shows the clinical characteristics of patients with severe obesity at baseline, 6 months and 12 months after RYGB. As expected, median BMI decreased significantly from 49.4 kg/m^2 at baseline, to 37.3 kg/m^2 at 6 months and 35.0 kg/m^2 at 12 months after surgery, which is accompanied by similar reductions in waist circumference. There were significant improvements in cardiovascular risk profile, which included significant decreases in blood pressure, triglycerides, apoB, HbA1c, fasting glucose, fasting insulin, and HOMA-IR, and an increase in HDL-C at 12 months after surgery. There was also a significant increase in cholesterol efflux capacity, PON1 activity and adiponectin levels, and significant decreases in ICAM-1, leptin and resistin levels 12 months after surgery. Some of the changes in clinical characteristics (systolic blood pressure, triglycerides, HbA1c, fasting glucose, fasting insulin, HOMA-IR) and biomarker levels (cholesterol efflux capacity, ICAM-1, adiponectin, and leptin) levels were statistically significant at 6 months after surgery.

Table 5.4. Clinical characteristics before surgery, and 6 and 12 months after surgery.

	Before Surgery (n=53)		6 months after surgery (n=42)			12 months after surgery (n=53)			Overall
	n	estimate	n	estimate	*P*-value*	n	estimate	*P*-value*	*P*-value†
BMI, kg/m²‡	53	49.4 (45.6–57.5)	43	37.3 (33.4–44.0)	**<0.001**	53	35.0 (30.3–38.5)	**<0.001**	**<0.001**
Waist circumference, cm	53	142 (17)	35	118 (16)	**<0.001**	53	106 (14)	**<0.001**	**<0.001**
SBP, mmHg‡	53	131 (120–146)	38	126 (109-–137)	**0.006**	52	119 (110–132)	**<0.001**	**<0.001**
DBP, mmHg	53	75.4 (13.8)	38	71.2 (14.8)	0.132	52	69.6 (10.5)	**0.002**	**0.041**
Total cholesterol, mmol/L‡	53	4.26 (3.79–5.28)	42	4.41 (3.55–5.49)	0.520	53	4.43 (3.73–5.14)	0.943	0.720
Triglycerides, mmol/L‡	53	1.46 (1.12–1.93)	41	1.29 (1.01–1.60)	**0.025**	53	1.08 (0.84–1.41)	**0.005**	**0.014**
HDL-C, mmol/L‡	53	1.03 (0.87–1.32)	42	1.17 (0.96–1.44)	0.070	53	1.29 (1.04–1.43)	**<0.001**	**<0.001**
LDL-C, mmol/L‡	52	2.44 (1.96–3.21)	41	2.56 (1.82–3.57)	0.817	53	2.43 (1.97–3.20)	0.547	0.468
ApoA-I, g/L‡	52	1.26 (1.15–1.40)	43	1.20 (1.10–1.42)	0.093	52	1.23 (1.11–1.38)	0.391	0.243
ApoB, g/L‡	53	0.80 (0.68–1.02)	43	0.77 (0.63–0.99)	0.139	53	0.73 (0.61–0.86)	**<0.001**	**0.002**
Cholesterol efflux capacity, %	53	12.94 (3.79)	43	14.28 (3.95)	**0.025**	53	16.03 (4.38)	**<0.001**	**<0.001**
PON1 activity, nmol/ml/min	53	67.0 (36.7–172.6)	43	69.7 (43.0–162.8)	0.683	53	83.0 (46.8–162.0)	**0.009**	**0.007**
HbA1c, mmol/L‡	51	45.4 (41.0–53.0)	44	38.4 (33.5–41.0)	**<0.001**	52	35.0 (32.3–37.5)	**<0.001**	**<0.001**
Fasting glucose, mmol/L‡	52	5.99 (5.13–6.78)	43	5.54 (4.68–6.19)	**0.006**	53	5.00 (4.66–5.82)	**0.002**	**0.001**
Fasting insulim, mU/L‡	52	18.44 (13.41–32.58)	42	9.46 (6.54–17.53)	**<0.001**	53	6.91 (4.75–12.29)	**<0.001**	**<0.001**
HOMA-IR‡	52	5.71 (3.35–8.61)	42	2.66 (1.65–4.30)	**<0.001**	53	1.54 (1.06–2.94)	**<0.001**	**<0.001**
HOMA-B‡	51	153 (64–258)	42	107 (72–168)	**0.047**	53	103 (69–159)	**0.032**	**0.006**
ICAM-1, ng/mL	53	199.0 (154.7–234.2)	42	163.7 (132.9–190.3)	**0.009**	53	136.8 (124.5–157.1)	**<0.001**	**<0.001**
Adiponectin, mg/L	52	3.28 (1.46)	42	4.39 (1.83)	**<0.001**	53	5.97 (2.67)	**<0.001**	**<0.001**
Leptin, ng/mL‡	52	70.3 (48.8–98.4)	42	25.8 (13.9–44.5)	**<0.001**	53	17.0 (8.5–36.0)	**<0.001**	**<0.001**
Resistin, ng/mL	52	15.1 (10.8–17.6)	42	12.6 (9.3–16.2)	0.099	53	9.4 (6.3–13.9)	**<0.001**	**<0.001**

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables.

*Data were compared with those at baseline using paired t-test.

†*P* values for change over time from baseline to 12 months after surgery (repeated measures ANOVA).

‡*P* values were estimated using ln-transformed data.

Change in HDL-associated miRNA levels after surgery

Table 5.5 shows the median and interquartile ranges of different HDL-associated miRNAs at baseline, 6 months, and 12 months after surgery; while Figure 5.2 shows the corresponding geometric mean and 95% confidence interval of these HDL-associated miRNAs. There were significant increases in HDL-associated miR-24, miR-222 and miR-223 levels, but not miR-126 level, at 12 months after surgery, in which the increase in HDL-associated miR-222 levels remained statistically significant at 6 months after correcting for multiple testing of two time-points. Although HDL-associated miR-24 showed a significant increase at 6 months, the increase was not statistically significant after correcting for multiple testing. For all these HDL-associated miRNAs, the change over time did not differ between groups divided based on gender, presence of type 2 diabetes, or statin therapy (all *P* for time interaction >0.05).

Among the HDL-associated miRNA levels that showed significant increase at 12 months after surgery, increase in HDL-associated miR-24 levels correlated strongly and positively with increase in HDL-associated miR-222 and miR-223 levels (r=0.732 and 0.577 respectively, both P<0.05). There was, however, no significant correlation between the changes in HDL-associated miR-222 and miR-223 levels (Table 5.6).

Table 5.5. Comparison of HDL-associated miR-223, miR-24, miR-126, and miR-222 levels at baseline before surgery, and 6 and 12 months after surgery.

	Before Surgery (n=53)		**6 months after surgery (n=42)**			**12 months after surgery (n=53)**			**Overall**
	n	**RQV/Protein (x10^{-5})**	**n**	**RQV/Protein (x10^{-5})**	***P*-value***	**n**	**RQV/Protein (x10^{-5})**	***P*-value***	***P*-value†**
miR-24	53	2.5 (1.0-71.8)	42	11.1 (2.2-106.1)	0.033	53	4.3 (2.0-73.3)	**0.004**	**0.023**
miR-126	53	6.5 (1.3-35.4)	42	8.2 (1.2-33.7)	0.943	53	6.5 (1.9-3.9)	0.062	0.112
miR-222	26	0.7 (0.4-1.2)	17	0.8 (0.5-1.1)	**0.016**	26	1.3 (0.9-2.0)	**0.003**	**0.002**
miR-223	53	18.4 (1.9-131.1)	42	50.0 (3.5-232.6)	0.071	53	24.4 (3.6-325.8)	**0.002**	**0.017**

Data are presented as median and interquartile range in the unit of RQV/protein and were ln-transformed before analysis.
†Data were compared with those at baseline using paired t-test.
**P*-values for change over time from baseline to 12 months after surgery (repeated measures ANOVA).

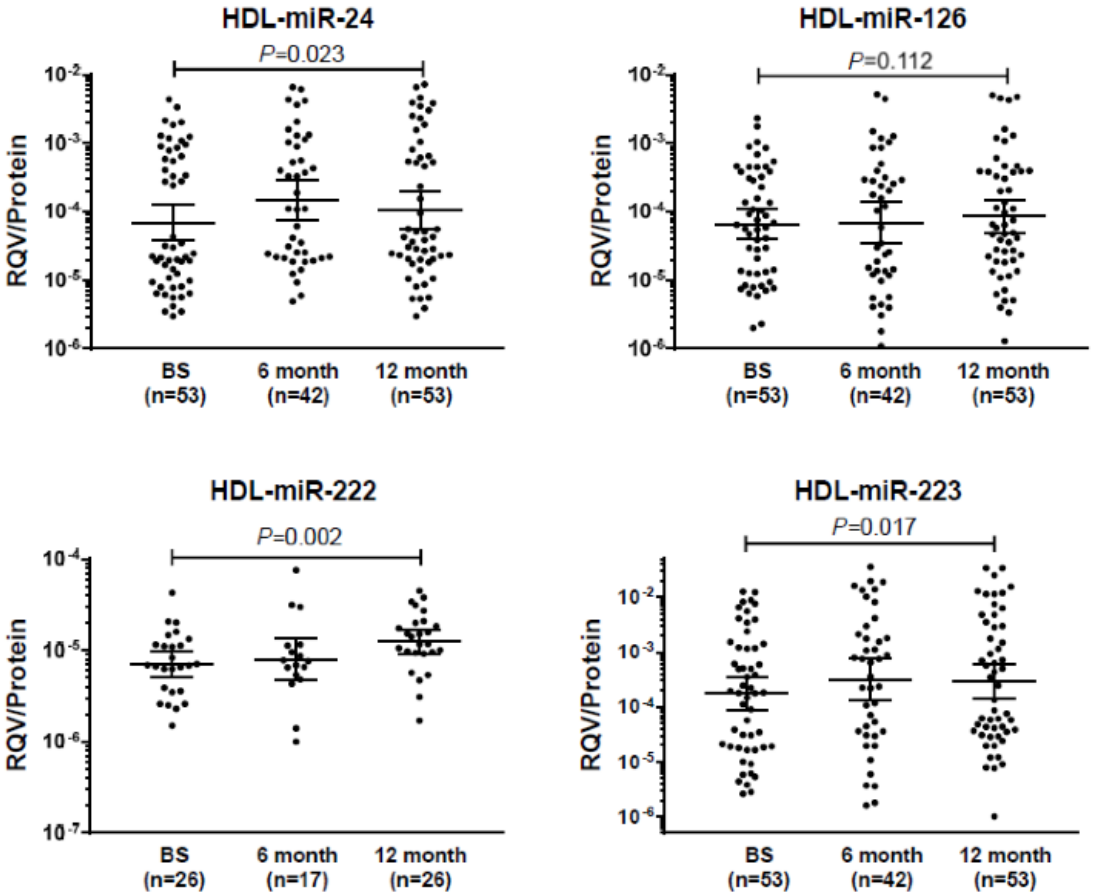


Figure 5.2. Distribution of different HDL-associated miRNA levels in obese subjects at baseline before surgery, and 6 and 12 months after surgery.

Data shown are geometric mean and 95% confidence interval with log scale on the y-axis. Data were compared using one-way univariate repeated measures ANOVA and participants with missing data at any time-points were excluded from the analysis.

Table 5.6. Bivariate Spearman correlation among changes in HDL-associated miR-24, miR-222 and miR-223 at 12 months after surgery.

	miR-24	***miR-222***	***miR-223***
6 months			
miR-24	-		
miR-222	**0.732‡**	-	
miR-223	**0.577‡**	0.354	-
12 months			
miR-24	-		
miR-222	**0.744‡**	-	
miR-223	**0.757‡**	0.342	-

*P<0.05, †P<0.01 and ‡P<0.001.

Correlation of changes in HDL-associated miRNA levels with changes in clinical characteristics

Although none of the HDL-associated miRNA levels correlated with BMI at baseline, changes in HDL-associated miR-24 levels at 12 months after surgery correlated positively with changes in BMI (r=0.309, P=0.024, Table 5.7). In fact, a significantly larger reduction in weight is observed in patients with sub-median change in HDL-associated miR-24 levels at 12 months after surgery (Table 5.8). A larger weight reduction was also observed among patients with decreased miR-24 (n=17) compared with patients with increased miR-24 (n=36) at 12 months after RYGB (-17.9 ± 5.7 vs -15.9 ± 5.8 kg/m^2, P=0.248) although statistical significance was not achieved. Weight reduction did not differ between groups divided using median baseline HDL-associated miRNA levels (Table 5.8).

Table 5.7. Spearman correlation between absolute changes in HDL-associated miRNAs and absolute changes in clinical characteristics at 12 months after surgery.

	∆miR-24	**∆miR-222**	**∆miR-223**
∆BMI, kg/m²	**0.309***	0.238	0.259
∆Waist circumference, cm	0.175	0.146	0.206
∆SBP, mmHg	**0.285***	0.232	0.089
∆DBP, mmHg	0.223	-0.030	0.153
∆Total cholesterol, mmol/L	0.034	0.142	-0.126
∆Triglycerides, mmol/L	**0.331***	-0.069	0.112
∆HDL-C, mmol/L	-0.230	-0.025	-0.117
∆LDL-C, mmol/L	-0.023	0.244	-0.115
∆ApoA-I, g/L	0.003	0.077	0.060
∆ApoB, g/L	0.085	-0.111	-0.060
∆Cholesterol efflux capacity, %	**0.326***	0.453	**0.349***
∆PON1 activity, nmol/ml/min	0.248	0.333	0.242
∆HbA1c, mmol/L	0.230	-0.123	0.187
∆Fasting glucose, mmol/L	0.023	-0.215	0.048
∆Fasting insulin, mU/L	-0.089	0.022	-0.057
∆HOMA-IR	-0.060	-0.025	-0.044
∆HOMA-B	-0.179	**0.433***	-0.185
∆ICAM-1, ng/mL	-0.147	0.109	-0.265
∆Adiponectin, mg/L	**-0.336***	-0.245	-0.246
∆Leptin, ng/mL	0.033	0.099	-0.089
∆Resistin, ng/mL	0.082	0073	0.029

Abbreviations: ApoA-I, apolipoprotein A-I; apoB, apolipoprotein B-100; BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycosylated haemoglobin; HDL-C, high-density lipoprotein cholesterol; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment for insulin resistance; ICAM-1, intercellular adhesion molecule-1; LDL-C low-density lipoprotein cholesterol; PON1, paraoxonase-1; SBP, systolic blood pressure.

*P<0.05, †P<0.01 and ‡P<0.001.

Table 5.8. Reduction in BMI according to HDL-associated miRNAs at baseline before surgery and their changes from baseline to 12 months after surgery.

	Reduction in BMI, kg/m^2	*P*-value
Before surgery		
miR-24		
<median (n=26)	15.2 (12.4-20.1)	0.803
≥median (n=27)	15.7 (13.2-19.7)	
miR-222		
<median (n=26)	14.1 (11.6-19.6)	0.300
≥median (n=27)	18.3 (13.1-20.2)	
miR-223		
<median (n=26)	15.7 (12.8-20.5)	0.669
≥median (n=27)	15.5 (13.0, 19.5)	
Change at 12 months		
Δ miR-24		
<median (n=26)	18.2 (14.0-20.7)	**0.015**
≥median (n=27)	14.0 (10.9-17.9)	
Δ miR-222		
<median (n=26)	16.1 (13.3-19.3)	0.762
≥median (n=27)	13.4 (11.4-20.8)	
Δ miR-223		
<median (n=26)	15.7 (13.3-20.1)	0.355
≥median (n=27)	14.5 (11.8-19.0)	

Data presented as median and interquartile range. Data was compared using Mann-Whitney U test.

There were significant positive correlations between changes in HDL-associated miR-24 and miR-223 levels with cholesterol efflux capacity (r=0.326, P=0.027 and r=0.349, P=0.017 respectively) (Table 5.7). The correlation between HDL-associated miR-222 and cholesterol efflux capacity did not achieve statistical significance (r=0.453, P=0.052) (Table 5.7). There were also trends for positive correlations between HDL-associated miR-24, miR-222 and miR-223 with PON1 activity which did not achieve statistical significance (r=0.248, P=0.074; r=0.333, P=0.097 and r=0.242, P=0.081 respectively). Similarly, a trend for negative correlation was

observed between changes in ICAM-1 and changes in HDL-associated miR-223 (r=-0.265, P=0.055) but not the other miRNAs.

Furthermore, HDL-associated miR-24 levels positively correlated with changes in triglyceride levels, and inversely with changes in adiponectin (r=0.331, P=0.015 and r=-0.336, P=0.015, Table 4) while changes in HDL-associated miR-222 positively correlated with changes in HOMA-B (r=0.433, P=0.035) (Table 5.7).

5.5 DISCUSSION

This is the first study to assess the changes in HDL-associated miRNAs following RYGB in patients with severe obesity. Here we demonstrate an alteration in miRNA signature in patients with severe obesity following bariatric surgery in tandem to the expected reduction in BMI and improvements in metabolic and glycaemic markers.

Following RYGB, there were significant increases in HDL-associated miR-24, miR-222 and miR-223, with positive correlations between miRNAs at baseline maintained at 12 months after surgery. The increase in HDL-associated miRNAs appear to indicate an overall increase in HDL function following surgery and this is supported by the positive correlations and trends observed with improvements in markers of HDL functionality such as cholesterol efflux capacity and PON1 activity. Both miR-222 and miR-223 are modulators of key components within the pathophysiology of cardiometabolic disease in obesity, and increases in these HDL-associated miRNAs could reflect enhancement of HDL's cardioprotective functions and explain at least some of the metabolic improvements that are observed following RYGB.

Multiple studies have suggested a role for miR-222 within the pathophysiological process underlying obesity and cardiometabolic disease. MiR-222 is closely related to glucose metabolism with increased expression of miR-222 in circulation being described in obesity,[7, 30] and within the adipose tissue of patients with gestational diabetes.[5] Higher levels of HDL-associated miR-222 have also been previously reported in patients with familial hypercholesterolaemia.[14] In our study, we have found a positive correlation between changes in HDL-miR-222 and HOMA-B,

suggesting it may have a role in improving glycaemia after bariatric surgery. The higher levels of HDL-miR-222 in obesity and diabetes shown in previous studies may reflect a compensatory rise in response to the underlying metabolic derangement rather than the cause. This hypothesis is consistent with miR-222 being shown to improve hyperglycaemia through proliferation of pancreatic beta cell in a previous study using murine models.[31] It is, however, important to point out that assessment of beta cell function using static measures of insulin and glucose is suboptimal and are influenced by factors such as insulin action, alterations in energy balance, and markers alterations in glucose and insulin before and after surgery.[32] Indeed, a reduction in HOMA-B was observed in our cohort despite improvements in all markers of glycaemia and therefore require further study with dynamic testing. Also, interestingly, in contrast with our findings, a previous study of patients who underwent RYGB found a significant post-surgical reduction of circulating plasma miR-222.[7] One potential explanation for these discrepant results may be that, despite a reduction in overall circulating miR-222, the amount transported by HDL is increased due to the enhanced uptake capacity. Further studies will be required to further assess this.

MiR-223 has an important role in the development and regulation of the immune system and is established as a potent regulator of inflammatory processes.[33] It has been previously associated with obesity with increased levels of visceral adipose tissue miR-223 being demonstrated.[34] Increased adipose tissue inflammation and marked systemic insulin resistance have been shown in miR-223 knockout mice on a high-fat diet.[35] Furthermore, the transfer of miR-223 from HDL has been shown to decrease ICAM-1 expression in endothelial cells.[15] Indeed, a negative correlation between HDL-associated miR-223 and serum ICAM-1 levels at baseline, and a negative trend between post-surgical changes were observed following RYGB. This provides support for enhanced HDL anti-inflammatory function following surgery, conferred in part, through the transfer of miR-223. Furthermore, we have also demonstrated a positive correlation between HDL-associated miR-223 and cholesterol efflux capacity and a trend with PON1 activity, contributing further to an overall picture of enhanced HDL function after RYGB.

Although miR-223 has previously been shown to predict the response to a non-surgical weight loss intervention with an 800-880 kcal/day hypocaloric diet,[36] miR-223 expression did not differ between patients who achieved supra- and sub-median reductions in BMI following RYGB. Furthermore, in contrast to our post-RYGB observation, a reduction in HDL-associated miR-223 had been demonstrated following high-protein diet-induced weight loss in patients with obesity.[29] This observed difference in impact on circulating miRNA can be explained by the difference in magnitude of reduction in weight and therefore adiposity following the dietary weight loss study[37] and this study. This is supported by both subcutaneous and omental adipose tissue being established sites of altered miRNA expression including miR-223 following weight loss intervention.[38, 39] There is likely also a difference in the impact on HDL functionality particularly its transporting capacity between dietary and surgical weight loss. Whilst cholesterol efflux capacity is shown to be increased post-RYGB in our study, studies on dietary weight loss are limited with no significant increase in cholesterol efflux capacity being noted following hypocaloric diet in the absence of exercise training.[40]

Elevated levels of miR-24 have been reported in abdominal adipose tissue of patients with obesity, and are positively correlated with percentage body fat.[10] Somewhat contrastingly, it has also been demonstrated that miR-24 has a role in modulating the expression of von Willibrand factor, where its levels are increased in type 2 diabetes when miR-24 levels are reduced through application of anti-miR-24, implicating a potential role in the risk of thrombotic cardiovascular events.[41] In the same study, miR-24 was also shown to be downregulated in endothelial cells in response to hyperglycaemia. It is therefore possible that the post-surgical increase in HDL-associated miR-24 observed in our cohort may result from glycaemic improvement as well as enhancement of HDL function, and may contribute to a reduction in cardiovascular risk following RYGB. Interestingly, although both HDL-associated miR-24 and adiponectin levels increased after RYGB, a negative correlation was seen between the two. Similarly, despite contrasting post-RYGB changes, positive correlations were observed between changes in miR-24 and change in BMI, triglyceride levels and systolic blood pressure. In keeping with this, a significantly larger reduction in BMI was also observed in patients with decreased or smaller increase in miR-24 at 12 months. This may reflect the complex

pathophysiological changes following RYGB, with differing relationships between miR-24 and changes in weight, adiposity, and glycaemia.

Despite increases after RYGB, both HDL-associated miR-222 and miR-223 were higher at baseline compared to healthy participants, with similar observations also noted with HDL-associated miR-126. Although the lower miRNA expression in healthy participants may seem unexpected given the post-surgical upregulation, there are two potential explanations for this observation. Firstly, the higher miR-126,[7] miR-222,[30] and miR-223[34] expressions in obesity is in keeping with previous studies and we postulate that this is likely triggered by underlying metabolic derangement which would be in line with the effect of miR-222 on glycaemia[31] and miR-223 on ICAM-1[15] shown in previous studies. The increase in HDL-associated miRNA after surgery is in keeping with the improvement in HDL function which may represent a dynamic process that driving metabolic improvements. It would be a great interest to see if this upregulation of HDL-associated miRNA then reverts to the levels observed in healthy participants once the process of metabolic correction is completed. Secondly, statin therapy had been shown to upregulate both miR-222 and miR-223 expression,[42] and a significant proportion of statin-use within our cohort with severe obesity is therefore likely to have contributed to the difference in miRNA expression.

Limitations to our study include the observational design and the small sample size particularly within the control group. As only patients who underwent RYGB were included in our study, these findings may therefore not be extended to other weight loss procedures. Further studies with a larger study population including other metabolic surgical procedures would allow for comparison of surgical procedures and confirm the findings in our study.

In conclusion, severe obesity is associated with altered HDL-associated miRNAs which is significantly changed following RYGB. The increase in expression of HDL-associated miRNAs following surgery may reflect an improvement in HDL function and may explain some of the cardiometabolic benefits observe following RYGB in severe obesity.

5.6 AUTHOR CONTRIBUTIONS

Study concept and design were by J.H. Ho, F. Tabet and H. Soran. J.H. Ho, S. Adam, Z. Iqbal, and S. Dhage undertook patient recruitment and clinical assessments. K.L. Ong, J.H. Ho, L.F. Cuesta Torres, and Y. Liu performed laboratory analyses. J.H. Ho and K.L Ong performed the data analyses and undertook interpretation of findings with F. Tabet and H. Soran. J.H. Ho produced the first draft and the final version with F. Tabet and H. Soran. S. Adam, Z. Iqbal, S. Dhage, B.J. Ammori, A.A. Syed, and P.N. Durrington provided critical review for important intellectual content.

5.7 ACKNOWLEDGEMENTS

We acknowledge support from National Institute of Health Research/Wellcome Trust Clinical Research Facility (Manchester, UK) and the Lipid Disease Fund.

5.8 DECLARATION OF INTERESTS

The authors of this manuscript have no conflict of interests to declare.

5.9 REFERENCES

1. Finucane MM, Stevens GA, Cowan MJ, et al. National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants. *Lancet.* 2011;377:557-567.

2. Ng M, Fleming T, Robinson M, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet.* 2014;384:766-781.

3. Adams TD, Davidson LE, Litwin SE, et al. Weight and Metabolic Outcomes 12 Years after Gastric Bypass. *N Engl J Med.* 2017;377:1143-1155.

4. Sjostrom L. Review of the key results from the Swedish Obese Subjects (SOS) trial - a prospective controlled intervention study of bariatric surgery. *J Intern Med.* 2013;273:219-234.

5. Deiuliis JA. MicroRNAs as regulators of metabolic disease: pathophysiologic significance and emerging role as biomarkers and therapeutics. *Int J Obes (Lond).* 2016;40:88-101.

6. Schroen B, Heymans S. Small but smart--microRNAs in the centre of inflammatory processes during cardiovascular diseases, the metabolic syndrome, and ageing. *Cardiovasc Res.* 2012;93:605-613.

7. Ortega FJ, Mercader JM, Catalan V, et al. Targeting the circulating microRNA signature of obesity. *Clin Chem.* 2013;59:781-792.

8. Hulsmans M, De Keyzer D, Holvoet P. MicroRNAs regulating oxidative stress and inflammation in relation to obesity and atherosclerosis. *FASEB J.* 2011;25:2515-2527.

9. Vickers KC, Remaley AT. MicroRNAs in atherosclerosis and lipoprotein metabolism. *Curr Opin Endocrinol Diabetes Obes.* 2010;17:150-155.

10. Nunez Lopez YO, Garufi G, Pasarica M, Seyhan AA. Elevated and Correlated Expressions of miR-24, miR-30d, miR-146a, and SFRP-4 in Human Abdominal Adipose Tissue Play a Role in Adiposity and Insulin Resistance. *Int J Endocrinol.* 2018;2018:7351902.

11. Arner E, Mejhert N, Kulyte A, et al. Adipose tissue microRNAs as regulators of CCL2 production in human obesity. *Diabetes.* 2012;61:1986-1993.

12. Wen D, Qiao P, Wang L. Circulating microRNA-223 as a potential biomarker for obesity. *Obes Res Clin Pract.* 2015;9:398-404.

13. Vickers KC, Remaley AT. Lipid-based carriers of microRNAs and intercellular communication. *Curr Opin Lipidol.* 2012;23:91-97.

14. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol.* 2011;13:423-433.

15. Tabet F, Vickers KC, Cuesta Torres LF, et al. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun.* 2014;5:3292.

16. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med.* 1977;62:707-714.

17. Rashid S, Genest J. Effect of obesity on high-density lipoprotein metabolism. *Obesity (Silver Spring).* 2007;15:2875-2888.

18. Zvintzou E, Skroubis G, Chroni A, et al. Effects of bariatric surgery on HDL structure and functionality: results from a prospective trial. *J Clin Lipidol.* 2014;8:408-417.

19. Heffron SP, Lin BX, Parikh M, et al. Changes in High-Density Lipoprotein Cholesterol Efflux Capacity After Bariatric Surgery Are Procedure Dependent. *Arterioscler Thromb Vasc Biol.* 2018;38:245-254.

20. Aron-Wisnewsky J, Julia Z, Poitou C, et al. Effect of bariatric surgery-induced weight loss on SR-BI-, ABCG1-, and ABCA1-mediated cellular cholesterol efflux in obese women. *J Clin Endocrinol Metab.* 2011;96:1151-1159.

21. Kjellmo CA, Karlsson H, Nestvold TK, et al. Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity. *J Clin Lipidol.* 2018;12:193-202.

22. Atkin SL, Ramachandran V, Yousri NA, et al. Changes in Blood microRNA Expression and Early Metabolic Responsiveness 21 Days Following Bariatric Surgery. *Front Endocrinol (Lausanne).* 2018;9:773.

23. Charlton-Menys V, Liu Y, Moorhouse A, Durrington PN. The robustness of the Roche 2nd generation homogenous HDL cholesterol (PEGME) method: assessment of the effect of serum sample storage for up to 8 years at -80 degrees C. *Clin Chim Acta.* 2007;382:142-144.

24. Charlton-Menys V, Liu Y, Durrington PN. Semiautomated method for determination of serum paraoxonase activity using paraoxon as substrate. *Clin Chem.* 2006;52:453-457.

25. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* 1985;28:412-419.

26. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol.* 2010;30:796-801.

27. Khera AV, Cuchel M, de la Llera-Moya M, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med.* 2011;364:127-135.

28. Hafiane A, Genest J. HDL-Mediated Cellular Cholesterol Efflux Assay Method. *Ann Clin Lab Sci.* 2015;45:659-668.

29. Tabet F, Cuesta Torres LF, Ong KL, et al. High-Density Lipoprotein-Associated miR-223 Is Altered after Diet-Induced Weight Loss in Overweight and Obese Males. *PLoS One.* 2016;11:e0151061.

30. Ortega FJ, Mercader JM, Moreno-Navarrete JM, et al. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care.* 2014;37:1375-1383.

31. Tsukita S, Yamada T, Takahashi K, et al. MicroRNAs 106b and 222 Improve Hyperglycemia in a Mouse Model of Insulin-Deficient Diabetes via Pancreatic beta-Cell Proliferation. *EBioMedicine.* 2017;15:163-172.

32. Bradley D, Magkos F, Klein S. Effects of bariatric surgery on glucose homeostasis and type 2 diabetes. *Gastroenterology.* 2012;143:897-912.

33. Taibi F, Metzinger-Le Meuth V, Massy ZA, Metzinger L. miR-223: An inflammatory oncomiR enters the cardiovascular field. *Biochim Biophys Acta.* 2014;1842:1001-1009.

34. Deiuliis JA, Syed R, Duggineni D, et al. Visceral Adipose MicroRNA 223 Is Upregulated in Human and Murine Obesity and Modulates the Inflammatory Phenotype of Macrophages. *PLoS One.* 2016;11:e0165962.

35. Zhuang G, Meng C, Guo X, et al. A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation. *Circulation.* 2012;125:2892-2903.

36. Milagro FI, Miranda J, Portillo MP, Fernandez-Quintela A, Campion J, Martinez JA. High-throughput sequencing of microRNAs in peripheral blood mononuclear cells: identification of potential weight loss biomarkers. *PLoS One.* 2013;8:e54319.

37. Wycherley TP, Brinkworth GD, Clifton PM, Noakes M. Comparison of the effects of 52 weeks weight loss with either a high-protein or high-carbohydrate diet on body composition and cardiometabolic risk factors in overweight and obese males. *Nutr Diabetes.* 2012;2:e40.

38. Macartney-Coxson D, Danielson K, Clapham J, et al. MicroRNA Profiling in Adipose Before and After Weight Loss Highlights the Role of miR-223-3p and the NLRP3 Inflammasome. *Obesity (Silver Spring).* 2020;28:570-580.

39. Kristensen MM, Davidsen PK, Vigelso A, et al. miRNAs in human subcutaneous adipose tissue: Effects of weight loss induced by hypocaloric diet and exercise. *Obesity (Silver Spring).* 2017;25:572-580.

40. Khan AA, Mundra PA, Straznicky NE, et al. Weight Loss and Exercise Alter the High-Density Lipoprotein Lipidome and Improve High-Density Lipoprotein Functionality in Metabolic Syndrome. *Arterioscler Thromb Vasc Biol.* 2018;38:438-447.

41. Xiang Y, Cheng J, Wang D, et al. Hyperglycemia repression of miR-24 coordinately upregulates endothelial cell expression and secretion of von Willebrand factor. *Blood.* 2015;125:3377-3387.

42. Li J, Chen H, Ren J, et al. Effects of statin on circulating microRNAome and predicted function regulatory network in patients with unstable angina. *BMC Med Genomics.* 2015;8:12.

CHAPTER 6: INSULIN RESISTANCE IS ASSOCIATED WITH IMPAIRED HDL FUNCTION AND ATHEROGENIC MODIFICATION OF LDL IN POLYCYSTIC OVARIAN SYNDROME

To be submitted

Author's contribution:

This is a completed cross-sectional observational study undertaken at Tallaght Hospital, Dublin. Samples were provided by Professor James Gibney.

The author undertook laboratory analyses under supervision apart from the basic lipid profile and androgen profile (made available by Professor James Gibney), and assays for oxidised phospholipids and lipoprotein(a) (undertaken at University of California San Diego), and anti-apoA-I IgG (undertaken at Geneva University Hospitals). The author researched the available literature, wrote the first draft, and completed the final draft with Dr Handrean Soran.

Insulin resistance is associated with impaired HDL function and atherogenic modification of LDL in polycystic ovarian syndrome

Jan Hoong Ho[1,2], Safwaan Adam[1,2], Yifen Liu[2], Shaishav Dhage[1,2], Sabrina Pagano[3], Xiaohong Yang[4], Paul N Durrington[2], Nicolas Vuilleumier[3], Sotirios Tsimikas[4], Handrean Soran[1,2]*, James Gibney[4]*

[1]Department of Medicine, Manchester University NHS Foundation Trust, Manchester, UK
[2]Lipid Research Group, The University of Manchester, Manchester, UK
[3]Department of Genetics and Laboratory Medicine, Division of Laboratory Medicine, Geneva University Hospitals, Geneva, Switzerland
[4]Sulpizio Cardiovascular Center, Division of Cardiovascular Medicine, University of California, San Diego, USA
[5]Department of Endocrinology and Diabetes, Tallaght Hospital, Tallaght, Ireland
*Contributed equally

Co-corresponding author:

Dr Handrean Soran MSc, MD, FRCP
Consultant Physician and Endocrinologist
E-mail: hsoran@aol.com; handrean.soran@mft.nhs.uk

6.1 ABSTRACT

Background and aims: Polycystic ovarian syndrome (PCOS) is associated with increased risk of cardiovascular disease (CVD). The aim of this study was to assess the association between PCOS and markers of HDL functionality and atherogenic lipoprotein modification.

Methods: This is a cross-sectional study of 104 women with PCOS (median age 29) and 103 BMI-matched healthy participants (median age 34). PCOS was defined using the NIH criteria. Measurement of lipid profile and glycaemic blood parameters were undertaken. Cholesterol efflux capacity, paraoxonase-1 (PON1) activity, and serum amyloid A (SAA) were measured as markers of HDL functionality. Oxidised LDL (OxLDL), lipoprotein-associated phospholipase A2 (LpPLA2), oxidised phoshopholipids on apolipoprotein B-100 (OxPL-apoB) and apolipoprotein(a) (OxPL-apo(a)), IgG and IgM antibodies to malondialdehyde-modified LDL (MDA-LDL) and apoB immune complexes (apoB-IC), and glycated apoB were used as markers of atherogenic modification of LDL. Anti-apoA-I IgG autoantibodies and positivity were also assessed.

Results: Patients with PCOS in the upper tertile of insulin resistance had impaired HDL functionality, with lower cholesterol efflux capacity and PON1 activity, and higher SAA, as well as increased markers of atherogenic modification of LDL, with higher levels of OxLDL, LpPLA2, small-dense LDL (sdLDL), and glycated apoB. Both BMI and insulin resistance are similarly associated with an adverse lipoprotein profile. OxPL-apoB, OxPL-apo(a), lipoprotein(a), and the IgG and IgM biomarkers did not differ between PCOS and controls. Anti-apoA-I antibody positivity did not appear to impact on markers of HDL function in this cohort.

Conclusions: Insulin resistance is a key determinant of decreased HDL functionality and increased oxidative modification and glycation of LDL in PCOS, which is likely to contribute to the increased CVD risk.

KEYWORDS

PCOS, insulin resistance, HDL functionality, cholesterol efflux, oxidised LDL, oxidised phospholipids.

6.2 INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder in women of reproductive age, characterised by menstrual dysfunction and hyperandrogenisim.[1, 2] It affects around 6-10% of women of reproductive age,[3] and the prevalence can increase to 20% depending on the diagnostic criteria used.[4] PCOS is recognised to confer an increased risk of cardiovascular disease (CVD), with increased prevalence of traditional risk factors such as obesity, impaired glucose tolerance, dyslipidaemia, and hypertension.[5, 6] Insulin resistance features prominently in PCOS, independent of obesity,[7] culminating in a long-term increased risk of incident metabolic syndrome and diabetes.[8] Overall, there is established evidence for the cardiometabolic burden associated with PCOS from early reproductive life.

The characteristic lipid profile associated with insulin resistance is commonly seen in PCOS, with elevated triglyceride and low high-density lipoprotein (HDL) cholesterol.[9] Furthermore, independent of lipid levels, there is also a shift towards small-dense low-density lipoprotein (sdLDL),[10] which is of increased atherogenicity and a marker for assessment of atherosclerotic CVD.[11] sdLDL circulates longer compared to the large low-density lipoprotein (LDL) and therefore more exposed to atherogenic modification.[12] sdLDL is also more prone to oxidative modification owing to its lipid composition[13] and low antioxidant content.[14] Oxidative modification of LDL leads to generation of proinflammatory and proatherogenic oxidation-specific epitopes such as oxidised phospholipids (OxPL), and malondialdehyde epitopes on LDL.[15] OxPL are key contributors to atherosclerosis and circulate bound preferentially to lipoprotein(a) [Lp(a)],[16] an independent and causal risk factor for CVD.[17] OxPL measured on apolipoprotein B-100 (apoB) containing lipoproteins (OxPL-apoB) is an established independent predictor of atherosclerotic CVD, and is thought to reflect Lp(a)-mediated effects in CVD.[15] In addition to oxidative modification, sdLDL is also more susceptible to glycation of apoB, further increasing its atherogenicity.[18]

Insulin resistance has been linked with oxidative modification and glycation of HDL,[19] along with marked alterations in the HDL lipidome, which in turn may impact on HDL functionality.[20] HDL has several cardioprotective functions, including anti-oxidation, anti-inflammation, anti-thrombosis, modulation of glycaemia, and reverse cholesterol transport. Consequently, impairment of HDL function has been linked with an

increased risk of CVD.[21, 22] A reduction in cholesterol efflux capacity was previously demonstrated in association with body mass index (BMI) and presence of metabolic syndrome in patients with PCOS.[23] The impact of PCOS on other functions of HDL, however, is not well established. Further to the impairment in HDL's atheroprotective function, modification of HDL may also result in formation of immunogenic epitopes and induce production of proatherogenic antibodies.[24] More recently, autoantibodies against apoA-I (anti-apoA-I IgG) have emerged as an independent predictor cardiovascular outcome.[25, 26] Whether these antibodies in turn results in modification of apoA-I thus impacting on HDL function is yet to be established.

In this study, we sought to assess the relationship between insulin resistance in PCOS and markers of HDL functionality and LDL oxidation and glycation. We also assessed the impact of anti-apoA-I IgG antibody positivity on HDL function in this cohort.

6.3 MATERIALS AND METHODS

Study design and participants

In this cross-sectional study, women with PCOS and control participants matched for body mass index (BMI) were recruited from the Endocrinology outpatient clinics at Adelaide and Meath Hospital (Tallaght, Dublin, Ireland).

PCOS was defined in accordance with the National Institute of Health (NIH) criteria as chronic oligomenorrhoea (less than nine menstrual cycles per year), and clinical and/or biochemical evidence of hyperandrogenism, following exclusion of other disorders causing the same phenotype.[1] Clinical hyperandrogenism included hirsutism (Ferriman-Gallway score more than 9), acne, or male pattern alopecia. Biochemical hyperandrogenism included elevated total testosterone, androstenedione or dehydroepiandrosterone sulphate (DHEAS) above the laboratory reference range. All patients had thyroid-stimulating hormone, free thyroxine, prolactin, luteinising hormone, follicle-stimulating hormone, oestradiol, and 17-hydroxyprogesterone measured to exclude other endocrine disorders. Patients with

clinical features of cortisol excess also underwent a 1mg overnight dexamethasone suppression test.

Control participants were recruited from the general population and comprised eumenorrhoeic healthy volunteers on no regular medications and with normal testosterone levels. This was done through advertisement in the study hospital, local schools and community centres. Study assessments were undertaken in the follicular phase of menstrual cycle.

Exclusion criteria included age < 18 or > 40 years, pregnancy, lactation, BMI < 19 kg/m^2 or > 50 kg/m^2, recent acute illness, chronic illnesses likely to influence results including diabetes mellitus, medications likely to influence results including hormonal contraception, antihypertensives, lipid-lowering medications, metformin, antiplatelet agents, anti-inflammatory agents, or non-prescription medications.

Ethical approval was obtained from the Research Ethics Committee of the Adelaide and Meath Hospital and St James' Hospital (Dublin, Ireland). Study assessments were undertaken in accordance with the 1964 Helsinki declaration. Written informed consent was obtained from all patients prior to participation in this study.

Laboratory analyses

All participants attended the Diabetes Day Centre at the Adelaide and Meath Hospital (Tallaght, Dublin, Ireland) on the study day following an overnight fast of at least 12 hours, with avoidance of excessive exercise and alcohol for at least 24 hours. Venous blood samples were obtained. Aliquots of serum and EDTA-plasma separated by centrifugation were stored frozen at -80°C until biochemical analyses could be performed at the end of study.

Androstenedione was measured using a radioimmune assay (Siemens, Munich, Germany). Sex hormone-binding globulin (SHBG) and DHEAS were measured using chemiluminescence immunoassays (Siemens, Munich, Germany). Insulin was measured using an electrochemiluminescence immunoassay, and glucose measured using an enzymatic hexokinase method on a Roche P Module Analyser

(Cobas Roche Diagnostics, West Sussex, UK). Glucose and insulin measurements were then used to calculate homeostatic model assessment of insulin resistance (HOMA-IR) and beta cell function (HOMA-B).[27] Total cholesterol, triglyceride, and HDL-C were measured using enzymatic colorimetric assays (Cobas Roche Diagnostics, West Sussex, UK). LDL cholesterol (LDL-C) was estimated using the Friedewald formula. sdLDL was assayed on a Randox Daytona+ analyser (Randox Laboratories, Crumlin, UK). Lp(a) measured using a validated chemiluminescent ELISA with monoclonal antibody LPA4 as previously described.[28, 29]

Biomarkers of HDL functionality

Cholesterol efflux capacity of HDL was determined using a previously validated method.[30, 31] The intra- and inter-assay coefficients of variation were 3.9% and 7.3% respectively. Briefly, J774A.1 cells were incubated with 0.2 µCi of radiolabelled ^{3}H-cholesterol in RPMI 1640 medium with 0.2% BSA at 37 °C in a 5% carbon dioxide humidified atmosphere. ABCA1 is upregulated using 0.3 mM C-AMP (8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium salt) for 4 hours and the cells incubated with 2.8% apoB-depleted serum using polyethylene glycol (PEG MW8000) for 4 hours. The cell media were then collected and cells dissolved in 0.5 ml 0.2 N NaOH to determine radioactivity. Cholesterol efflux was expressed as the percentage of radioactivity in the medium from the radioactivity in the cells and medium collectively:

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

Serum paraoxonase (PON1) activity was measured using paraoxon (O,O-diethyl O-(4-nitrophenyl) phosphate) as a substrate (Sigma-Aldrich, St Louis, USA).[32] The rate of p-nitrophenol generation was determined at 405nm on a Randox Daytona+ analyser (Randox Laboratories, Crumlin, UK). Serum amyloid A (SAA) was assayed using ELISA kits from ThermoFisher Scientific (Loughborough, UK) and myeloperoxidase (MPO) using an in-house sandwich ELISA using anti-human MPO capture and detection antibodies from R&D Systems Ltd (Abingdon, UK).

Anti-apoA-I IgG antibody titre and positivity

Anti-apoA-I IgG autoantibodies were measured using methods as previously described.[25, 26] Briefly, Maxisorb plates (Nunc, Glostrup, Denmark) were coated with purified human-derived delipidated apoA-I and then blocked with 2% bovine serum albumin (BSA) in a phosphate buffer solution (PBS) at 37°C. Samples dilated to 1:50 in PBS/BSA 2% solution were added to both coated and non-coated wells to allow assessment of individual non-specific binding. Following this, 50μl of alkaline phosphatase-conjugated anti-human IgG signal antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:1000 in PBS/BSA 2% solution was added to each well and incubated for 1 hour before 50μl of phosphatase substrate p-nitrophenyl phosphate disodium (Sigma-Aldrich, St Louis, MO, USA) dissolved in diethanolamine buffer (pH 9.8) was then added. Samples were tested in duplicates and optical density (OD) was determined at 405 nm (Molecular Devices™ Versa Max, Sunny Vale, CA, USA). The corresponding non-specific binding was subtracted from mean absorbance.

The cut off for anti-apoA-1 IgG positivity was determined as previously described.[25, 26] The upper reference range was derived from the 97.5th percentile of the reference population of 140 healthy blood donors and this corresponded with an OD cut off of 0.64. An index consisting the ratio between OD of the sample and OD of the positive control, expressed as a percentage, is further calculated to to minimise the impact of inter-assay variation. The index value of 37% corresponded with the 97.5th percentile of the normal distribution. Samples with an absorbance value greater than 0.64 OD and an index value greater than 37% were considered positive for elevated anti-apoA-I IgG levels.

Markers of LDL modification

Oxidised LDL levels in plasma was measured using Mercodia ELISA kits (Diagenics Ltd, Milton Keynes, UK), and LpPLA2 using USCN Lifescience ELISA kits (Hölzel Diagnostika Handels GmbH, Köln, Germany). Glycated apoB was measured using

Glycacor ELISA kits (Exocell, Philadelphia, USA) which uses mouse monoclonal antibody ES12 which detects a specific epitope on glycated apoB.

OxPL-apoB and OxPL-apo(a) levels were measured by means of a chemiluminescent immunoassay using murine monoclonal antibody E06.[15, 33, 34] E06 recognises the phosphocholine (PC) group on oxidised but not native phospholipids and similarly recognises the PC covalently bound to bovine serum albumin (BSA) in PC-BSA. A 1:50 dilution of plasma in 1% BSA in TBS was added to microtitre wells coated with the apoB specific monoclonal antibody MB47. A saturating amount of apoB was added to each well and therefore equal numbers of apoB particles are captured in each well. Biotinylated E06 was then added to determine the content of OxPL-apoB, which reflects the absolute content of OxPL per constant amount of captured apoB lipoprotein, independent of plasma levels of apoB or LDL-C. OxPL-apo(a) levels were measured in a similar manner to Ox-PL-apoB, apart from the use of LPA4 capture antibody to detect apo(a).[35] The microtitre wells were coated with LPA4 antibody, and plasma added to saturate the plate with apo(a). Biotinylated E06, which does not react with LPA4, is then added to content of OxPL-apo(a).

IgG and IgM MDA-LDL and apoB-IC levels were determined as previously described.[36, 37] For IgG and IgM MDA-LDL, a 1:200 dilution of plasma was added to microtitre wells coated with MDA-LDL (5µg/ml) and alkaline phosphatase-labeled goat anti-human IgG and IgM (Sigma-Aldrich, St Louis, USA) was then added to detect binding to MDA-LDL. For IgG and IgM apoB-IC, murine monoclonal antibody MB47 was plated to bind a saturating amount of human apoB. A 1:50 dilution of plasma was added and alkaline phosphatase-labeled goat anti-human IgG and IgM (Sigma-Aldrich, St Louis, USA) used to detect IgG or IgM autoantibodies bound to the captured apoB (apoB-IC) as above.

Statistical analyses

Statistical analyses were performed using SPSS for Mac (Version 23.0, IBM SPSS Statistics, Armonk, New York, USA) and figures were produced using GraphPad Prism for Mac (Version 8.00, GraphPad Software, La Jolla California, USA). Results

are presented as mean and standard deviation for parametric or as median and interquartile range for non-parametric variables. Comparison of variables between PCOS and controls was undertaken using the independent samples t-test for parametric and the Mann-Whitney U test for non-parametric variables. Normality of data distribution was assessed using the Shapiro-Wilk test and visualisation of histograms. Patients with PCOS were sub-divided into three groups based on tertiles of insulin resistance estimated using the HOMA-IR equation. Correlations between variables were assessed using bivariate Spearman's analyses. *P*-values < 0.05 were considered to be statistically significant.

6.4 RESULTS

Patient characteristics and lipid profile

The clinical characteristics and biochemical profile of the 104 patients with PCOS and 103 BMI-matched control participants are summarised in Table 6.1. Androstenedione and DHEAS levels were significantly higher among patients with PCOS, as expected. Overall, compared to controls, the cohort with PCOS also had a lower HDL-C, higher triglycerides and an unfavourable glycaemic profile with higher fasting insulin levels and insulin resistance estimated by HOMA-IR (Table 6.1). There were no significant differences in total cholesterol, LDL-C, apoB or apoA-I.

In patients with PCOS in the upper HOMA-IR tertile, there were significant differences in all variables apart from total cholesterol when compared to the lower HOMA-IR tertile (Table 2). Patients with PCOS whose HOMA-IR was in the upper tertile displayed significantly higher triglycerides, apoB and sdLDL with a lower HDL-C compared to the control cohort. Conversely, the lipid profile within the lower HOMA-IR tertile was more favourable compared to the control cohort, with lower triglycerides, LDL-C, and sdLDL, along with higher HDL-C and ApoA-I (Table 6.2).

Table 6.1. Characteristics of patients with polycystic ovarian syndrome and controls.

	PCOS (n=104)	Controls (n=103)	*P*-value
Clinical characteristics			
Age, y	29 (24–36)	34 (27–37)	**<0.001****
BMI, kg/m²	32.9 (25.7–38.5)	31.1 (27.6–35.5)	0.473
Lipid profile			
Total cholesterol, mmol/L	4.4 (0.8)	4.6 (0.8)	0.166
Triglyceride, mmol/L	1.1 (0.8–1.5)	0.9 (0.7–1.3)	**0.047***
HDL-C, mmol/L	1.34 (1.16–1.65)	1.49 (1.26–2.09)	**0.010***
LDL-C, mmol/L	2.5 (0.4)	2.6 (0.8)	0.258
ApoB, g/L	0.71 (0.17)	0.71 (0.16)	0.895
ApoA-I, g/L	1.52 (1.35–1.84)	1.46 (1.22–1.77)	0.203
Markers of glycaemia			
Fasting glucose, mmol/L	4.7 (4.5–5.0)	4.6 (4.4–4.9)	0.141
Fasting insulin, pmol/L	13.6 (8.0–19.3)	9.5 (5.7–14.8)	**0.001****
HOMA-IR	2.83 (1.61–4.48)	2.02 (1.13–3.10)	**0.001****
HOMA-B	198.6 (140.9–335.6)	165.4 (113.9–259.2)	**0.003****
Androgen profile			
Androstenedione, nmol/L	16.6 (13.0–21.0)	9.3 (8.0–12.6)	**<0.001****
DHEAS, µmol/L	6.1 (4.4–8.2)	4.6 (3.4–6.2)	**<0.001****
SHBG, mmol/L	28.5 (22.8–47.0)	41.0 (29.0–60.4)	**<0.001****

Data presented as mean and standard deviation for parametric and mean and interquartile range for non-parametric variables.

Comparison of variables with controls was preformed using independent samples t-test for parametric and Mann-Whitney U test for non-parametric variables. **P*<0.05, ** *P*<0.01

Abbreviations: BMI, body mass index; DHEAS, dehydroepiandrosterone sulphate; HDL-C, high-density lipoprotein cholesterol; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein cholesterol; SHBG, sex hormone binding globulin.

Table 6.2. Lipid profile in HOMA-IR tertiles in patients with PCOS and controls.

	Controls (n=103)	PCOS (n=104)		
		HOMA-IR		
		Lower tertile	Middle tertile	Upper tertile
Total cholesterol, mmol/L	4.4 (0.8)	4.4 (0.6)	4.3 (0.8)	4.6 (1.0)
Triglyceride, mmol/L	1.1 (0.8–1.5)	**0.8 (0.6–0.9)***	1.1 (0.8–1.4)	**1.5 (1.1–1.9)**, ††**
HDL-C, mmol/L	1.34 (1.16–1.65)	**1.75 (1.53–2.04)****	1.29 (1.14–1.53)	**1.16 (0.95–1.27)**, ††**
LDL-C, mmol/L	2.5 (0.4)	**2.3 (0.5)***	2.4 (0.8)	2.7 (0.8)†
ApoB, g/L	0.71 (0.17)	0.65 (0.15)	0.69 (0.16)	**0.76 (0.19)*, ††**
ApoA-I, g/L	1.52 (1.35–1.84)	**1.73 (1.53–2.08)****	1.47 (1.34–1.65)	1.40 (1.21–1.55)†
sdLDL, mg/dL	20.9 (14.6–29.0)	**15.3 (11.3–20.1)****	17.0 (11.7–25.4)	**24.8 (16.8–35.0)*, ††**
Glycated apoB, mg/dL	3.48 (3.20–3.96)	3.51 (3.27–3.70)	**3.83 (3.44–4.10)***	**4.02 (3.63–4.33)**, ††**

Data presented as mean and standard deviation for parametric and mean and interquartile range for non-parametric variables. Comparison of variables between groups was preformed using independent samples t-test for parametric and Mann-Whitney U test for non-parametric variables.
*P<0.05, ** P<0.01 compared to control
†P<0.05, ††P<0.01 compared to lower tertile

Abbreviations: ApoA-I, apolipoprotein A-I; apoB, apolipoprotein B-100; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein cholesterol; sdLDL, small dense LDL.

Markers of HDL functionality

Although cholesterol efflux capacity did not differ significantly between the overall PCOS and control cohorts [14.1 (13.2–15.5) vs 14.5 (13.0–16.0) %, *P*=0.647], it was significantly lower in the upper HOMA-IR tertile compared to the lower HOMA-IR tertile [13.7 (12.4–14.6) vs 14.9 (13.6–17.0) %, *P*=0.003] with a trend for lower cholesterol efflux capacity with increasing insulin resistance (Figure 6.1). Cholesterol efflux capacity was also lower in the upper HOMA-IR tertile when compared to the control cohort although this did not achieve statistical significance [13.7 (12.4–14.6) vs 14.5 (13.0–16.0) %, *P*=0.063].

PON1 activity was significantly lower in the overall PCOS cohort compared to the control cohort [102.1 (51.7–150.9) vs 131.6 (89.5–195.1) nmol/ml/min, *P*=0.002], as well as within the upper HOMA-IR tertile compared to the lower HOMA-IR tertile [77.2 (48.2–129.2) vs 112.9 (54.0–175.4) nmol/ml/min, *P*=0.043] (Figure 1). Higher SAA levels were observed in the upper HOMA-IR tertile compared to the control cohort [41.1 (24.7–65.0) vs 22.5 (11.8–48.9) µg/mL, *P*=0.022], with a trend also observed when compared to the lower HOMA-IR tertile which did not achieve statistical significance [41.1 (24.7–65.0) vs 29.8 (15.1–44.4) µg/mL, *P*=0.069]. MPO levels were not significantly different across tertiles of insulin resistance among patients with PCOS and the control cohort.

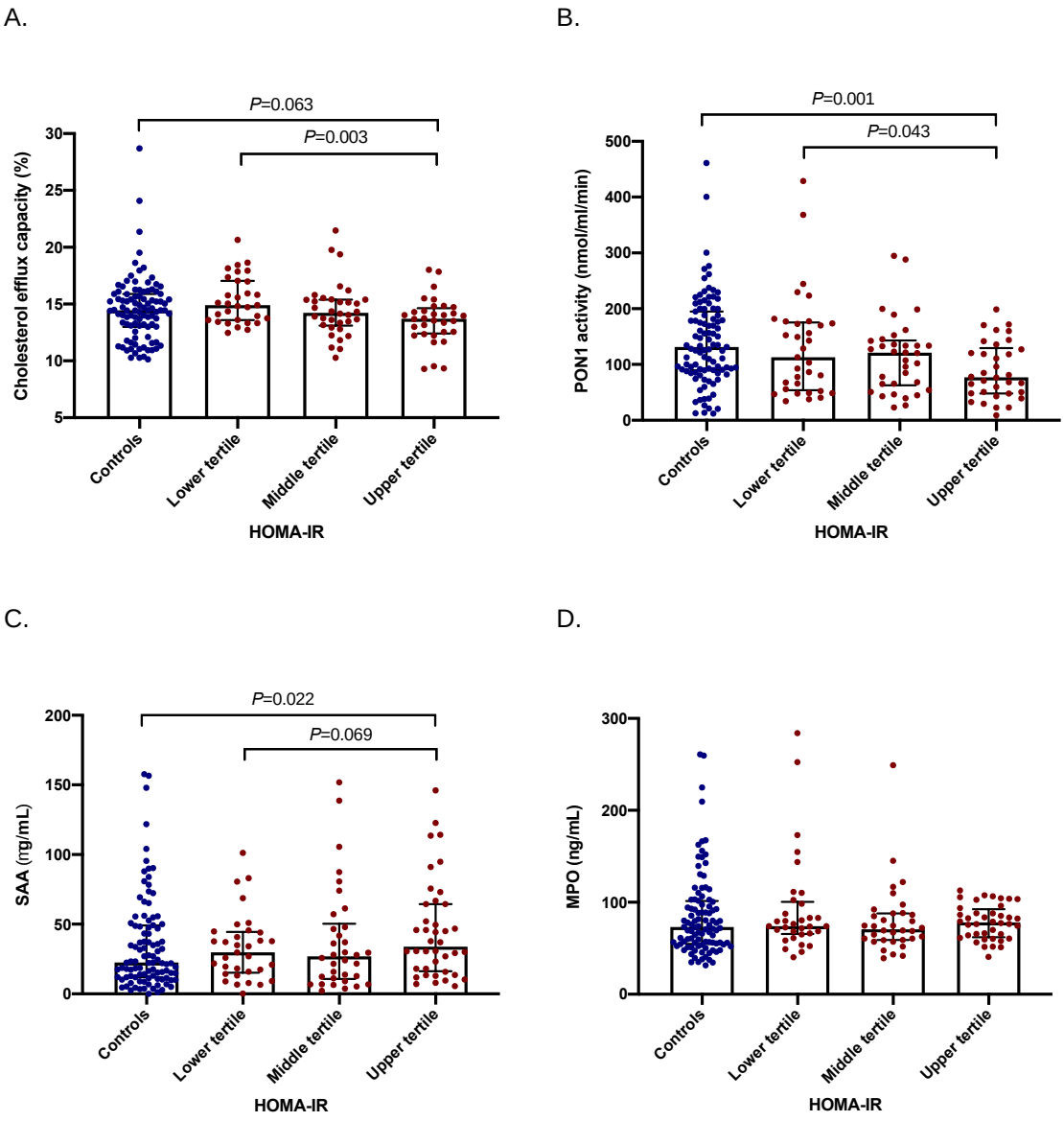


Figure 6.1. Markers of HDL function in PCOS and controls.

A) Cholesterol efflux capacity, B) PON1 activity, C) Serum amyloid A, and D) MPO mass according to tertiles of HOMA-IR in patients with PCOS compared to controls. Data presented as mean and standard deviation for parametric and mean and interquartile range for non-parametric variables.

Abbreviations: MPO, myeloperoxidase; PON1, paraoxonase-1; SAA, serum amyloid A.

Markers of LDL modification

Glycated apoB was higher in the overall PCOS cohort compared to controls [3.72 (3.34–4.07) vs 3.48 (3.20–3.96) mg/dL, *P*=0.020]. Glycated apoB also showed an increasing trend going up the HOMA-IR tertiles [3.51 (3.27–3.70) vs 3.83 (3.44–4.10) vs 4.02 (3.63–4.33) mg/dL] (Table 6.2).

Both OxLDL and LpPLA2 were not significantly different in the overall PCOS and control cohorts [77.4 (48.0–103.2) vs 74.8 (47.6–89.5) ng/mL, *P*=0.318; and 1.52 (1.39–1.67) vs 1.53 (1.37–1.70) µg/mL, *P*=0.924 respectively]. Both variables, however, were significantly higher in the upper HOMA-IR tertile compared to both the lower HOMA-IR tertile [91.6 (58.8–120.9) vs 67.2 (20.1–86.3) ng/mL, *P*=0.016; and 1.66 (1.48–1.84) vs 1.48 (1.39–1.60) µg/mL, *P*=0.004 respectively] and control cohort [91.6 (58.8–120.9) vs 74.8 (47.6–89.5) ng/mL, *P*=0.013; and 1.66 (1.48–1.84) vs 1.53 (1.37–1.70) µg/mL, *P*=0.015 respectively] (Figure 6.2).

There were, no significant differences in IgG and IgM MDA-LDL, and IgG and IgM apoB-IC across HOMA-IR tertiles and controls (Figure 6.2). Likewise, similar levels of both OxPL-apoB and OxPL-apo(a) were observed across the HOMA-IR tertiles and in the control cohort.

OxPL-apoB and OxPL-apo(a) were both strongly and positively correlated with Lp(a) (Spearman's ρ=0.838, *P*<0.001 and Spearman's ρ=0.875, *P*<0.001 respectively) (Table 6.3). OxPL-apoB, OxPL-apo(a), and Lp(a) did not differ between PCOS and controls. There were positive correlations between all the autoantibodies and immune complexes to MDA-LDL (Spearman's ρ=0.203–0.654, all *P*<0.050) (Table 6.3). No significant correlations, however, were observed between these indirect markers of LDL oxidation and OxLDL.

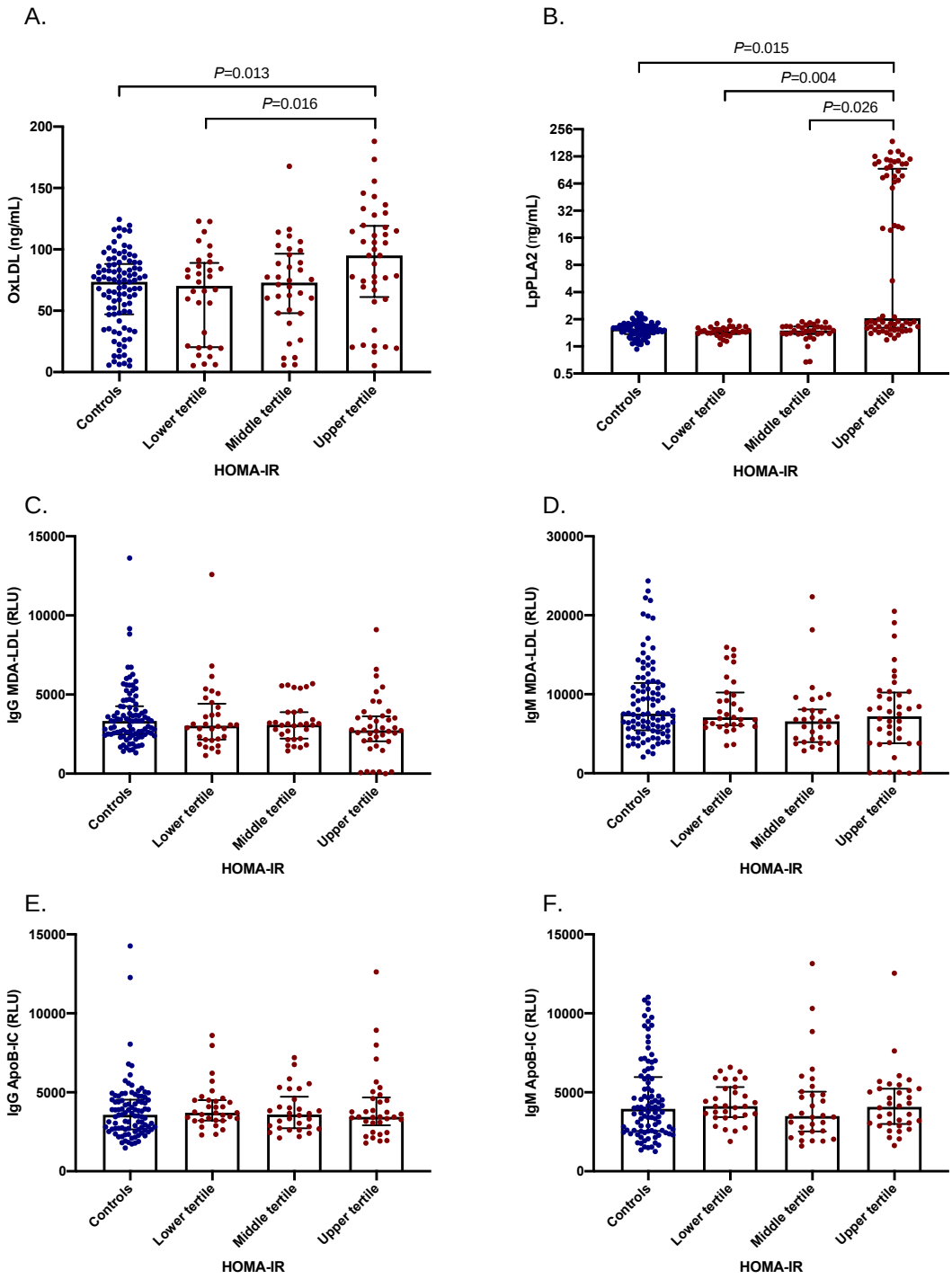


Figure 6.2. Markers of lipoprotein oxidation in PCOS and controls.

Levels of A) OxLDL, B) LpPLA2, C) IgG MDA-LDL, D) IgM MDA-LDL, E) IgG apoB-IC, and F) IgM apoB-IC according to tertiles of HOMA-IR in patients with PCOS compared to controls. Data presented as mean and standard deviation for parametric and mean and interquartile range for non-parametric variables.

Abbreviations: IC, immune complex; LDL, low-density lipoprotein; LpPLA2, lipoprotein-associated phospholipase A2; MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL

Table 6.3. Bivariate Spearman correlation between lipoprotein(a), oxidised phospholipids, and autoantibodies and immune complexes to oxidised LDL in patients with PCOS.

	Lp(a)	**OxPL-apoB**	**OxPL-apo(a)**	**IgG MDA-LDL**	**IgM MDA-LDL**	**IgG apoB-IC**	**IgM apoB-IC**	**OxLDL**
Lp(a), mg/dL	-							
OxPL-apoB, nM	**0.838****	-						
OxPL-apo(a), nM	**0.875****	**0.885****	-					
IgG MDA-LDL, RLU	0.041	0.153	0.089	-				
IgM MDA-LDL, RLU	0.016	0.032	-0.041	**0.203***	-			
IgG IC, RLU	-0.085	-0.008	-0.059	**0.493****	0.208*	-		
IgM IC, RLU	0.121	0.119	0.115	**0.214***	0.654**	0.225*	-	
OxLDL, ng/mL	0.109	0.092	0.081	0.019	0.078	-0.023	0.079	-

Abbreviations: Apo(a), apolipoprotein(a); IC, immune complex; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100.

*P<0.05, **P<0.01

Anti-apoA-I IgG autoantibodies

Both the prevalence of anti-apoA-I IgG positivity and the titre of anti-apoA-I IgG autoantibodies (expressed in OD) did not differ between the overall PCOS and control cohorts [33 (31.7%) vs 27 (26.2%), *P*=0.301; and 0.50 (0.39–0.73 vs 0.51 (0.34–0.66), *P*=0.365].

MPO levels were higher among patients with anti-apoA-I IgG positivity within the PCOS cohort [82.2 (69.3–106.4) vs 73.3 (62.3–85.6) ng/mL, *P*=0.017) but there were no significant differences in the other HDL functionality markers. IgG MDA-LDL and IgG apoB-IC were also both higher in the anti-apoA-I IgG positive cohort [3721 (2782–5431) vs 2898 (2169–3456) RLU, *P*=0.001; and 4398 (3397–6121) vs 3425 (2886–4043) RLU, *P*=0.002 respectively]. The other markers of LDL oxidation, including IgM MDA-LDL and apoB-IC, did not differ between the two groups, and although LpPLA2 appeared marginally higher in the antibody positive group, it did not achieve statistical significance (Table 6.4).

Markers of glycaemia were comparable between both groups.

Table 6.4. Comparing markers of HDL functionality, lipoprotein oxidation, and glycaemia in PCOS and apoA-I IgG positivity.

	Anti-apoA-I IgG positive (n=33)	Anti-apoA-I IgG negative (n=71)	*P*-value
Markers of HDL functionality			
ApoA-I, g/l	1.53 (1.22–1.84)	1.51 (1.36–1.84)	0.850
Cholesterol efflux capacity, %	14.8 (13.5–15.9)	13.9 (13.0–15.4)	0.112
PON1 activity, nmol/ml/min	92.8 (54.1–161.8)	109.1 (50.9–146.3)	0.746
SAA, µg/mL	29.3 (15.9–51.0)	32.4 (13.5–49.0)	0.881
MPO, ng/mL	82.2 (69.3–106.4)	73.3 (62.3–85.6)	**0.017***
Markers of lipoprotein oxidation			
OxLDL, ng/mL	77.5 (59.5–100.1)	76.1 (30.6–105.8)	0.545
LpPLA2, µg/mL	1.58 (0.28)	1.51 (0.24)	0.273
OxPL-apoB, nM	1.14 (0.66–2.98)	1.28 (0.75–4.15)	0.389
IgG MDA-LDL, RLU	3721 (2782–5431)	2898 (2169–3456)	**0.001****
IgM MDA-LDL, RLU	7230 (5671–12824)	7106 (5527–9594)	0.337
IgG apoB-IC, RLU	4398 (3397–6121)	3425 (2886–4043)	**0.002****
IgM apoB-IC, RLU	5003 (2818–5899)	3898 (3045–4840)	0.228
Markers of glycaemia			
Fasting glucose, mmol/L	4.6 (4.4–5.2)	4.7 (4.5–5.0)	0.533
Fasting insulin, pmol/L	14.6 (8.3–17.8)	12.7 (7.4–20.5)	0.918
HOMA-IR	2.97 (1.71–3.95)	2.47 (1.44–4.60)	0.831
HOMA-B	184.4 (152.7–337.8)	208.8 (137.8–326.7)	0.991

Data presented as mean and standard deviation for parametric and mean and interquartile range for non-parametric variables. Comparison of variables between groups was preformed using independent samples t-test for parametric and Mann-Whitney U test for non-parametric variables.

*P<0.05, ** P<0.01

Abbreviations: ApoA-I, apolipoprotein A-I; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment for insulin resistance; IC, immune complex, LpPLA2, lipoprotein associated phospholipase A2; MDA-LDL, malondialdehyde-modified LDL; MPO, myeloperoxidase; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; PON1, paraoxonase-1; SAA, serum amyloid A.

Correlation with BMI and insulin resistance

There were positive correlations between BMI and HOMA-IR in both patients with and without PCOS, with a stronger correlation coefficient observed in patients with PCOS (Spearman's ρ=0.709, *P*<0.001 and Spearman's ρ=0.550, *P*<0.001 respectively).

In patients with PCOS, both BMI and HOMA-IR correlated directly with triglycerides (Spearman's ρ=0.438, *P*<0.001 and Spearman's ρ=0.532, *P*<0.001 respectively), and inversely with HDL-C (Spearman's ρ=-0.593, *P*<0.001 and Spearman's ρ=-0.675, *P*<0.001 respectively) and ApoA-I (Spearman's ρ=-0.450, *P*<0.001 and Spearman's ρ=-0.471, *P*<0.001 respectively) (Table 6.5).

Likewise, BMI and HOMA-IR also correlated positively with sdLDL (Spearman's ρ=0.278, *P*<0.001 and Spearman's ρ=0.377, *P*<0.001 respectively) and glycated apoB (Spearman's ρ=0.279, *P*<0.001 and Spearman's ρ=0.359, *P*<0.001 respectively) (Table 6.5). sdLDL and glycated apoB were also positively correlated with each other (Spearman's ρ=0.556, *P*<0.001).

Cholesterol efflux capacity correlated negatively with both BMI (Spearman's ρ=-0.244, *P*=0.014) and insulin resistance (Spearman's ρ=-0.299, *P*=0.003) but similar associations with PON1 activity did not achieve statistical significance (Spearman's ρ=-0.185, *P*=0.064 and Spearman's ρ=-0.195, *P*=0.051 respectively) (Table 6.5). A positive correlation was observed between SAA and BMI (Spearman's ρ=0.332, *P*=0.001), but did not achieve statistical significance with insulin resistance (Spearman's ρ=0.175, *P*=0.080).

Table 6.5. Bivariate Spearman correlation between BMI, markers of glycaemia, lipid profile, and markers of HDL function in patients with PCOS.

	BMI	HOMA-IR	Fasting glucose	Fasting insulin
Lipid profile				
Total cholesterol, mmol/L	0.092	0.057	0.039	0.069
Triglyceride, mmol/L	**0.438****	**0.532****	**0.415****	**0.502****
HDL-C, mmol/L	**-0.593****	**-0.675****	**-0.341****	**-0.671****
LDL-C, mmol/L	0.191	0.178	0.020	**0.205***
Lipoprotein(a), mg/dL	-0.048	-0.065	**-0.266***	-0.032
ApoB, g/L	0.135	0.204*	0.110	**0.205***
ApoA-I, g/L	**-0.450****	**-0.471****	-0.128	**-0.474****
sdLDL, mg/dL	**0.278****	**0.377****	**0.224***	**0.365****
Glycated apoB, mg/dL	**0.279****	**0.359****	0.104	**0.382****
Markers of HDL functionality				
Cholesterol efflux capacity, %	**-0.244***	**-0.299***	-0.118	**-0.313****
PON1 activity, nmol/ml/min	-0.185	-0.195	-0.177	-0.168
SAA, µg/mL	**0.332****	0.175	0.099	0.168
MPO, ng/mL	0.172	0.022	0.123	0.027
Markers of lipoprotein oxidation				
OxLDL, ng/mL	**0.244***	**0.254***	0.112	**0.254***
LpPLA2, µg/mL	**0.325****	**0.291****	0.017	**0.306****
OxPL-apoB, nM	-0.059	-0.091	**-0.239***	-0.063
OxPL-apo(a), nM	-0.107	-0.132	**-0.297****	-0.102
IgG MDA-LDL, RLU	0.065	0.012	-0.122	0.021
IgM MDA-LDL, RLU	-0.033	-0.058	0.028	-0.082
IgG apoB-IC, RLU	-0.110	-0.118	-0.112	-0.103
IgM apoB-IC, RLU	-0.070	-0.135	-0.017	-0.146

Abbreviations: Apo(a), apolipoprotein(a); apoA-I, apolipoprotein A-I; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment for insulin resistance; IC, immune complex, LpPLA2, lipoprotein associated phospholipase A2; MDA-LDL, malondialdehyde-modified LDL; MPO, myeloperoxidase; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; PON1, paraoxonase-1; SAA, serum amyloid A; sdLDL, small dense LDL.

Both OxLDL and LpPLA2 correlated positively with BMI (Spearman's ρ=0.244, *P*=0.013 and Spearman's ρ=0.325, *P*=0.001 respectively) and insulin resistance (Spearman's ρ=0.254, *P*=0.011 and Spearman's ρ=0.291, *P*=0.003 respectively) but similar associations were not observed with the other markers of lipoprotein oxidation.

Overall, BMI and insulin resistance are both associated with an adverse lipoprotein profile (higher triglyceride, sdLDL, glycated apoB, OxLDL, LpPLA2, SAA and lower HDL-C, apoA-I, and cholesterol efflux capacity), which is also associated more with fasting insulin than fasting glucose levels (Table 6.5).

6.5 DISCUSSION

This study is the first study to simultaneously assess markers of HDL function, markers of oxidative modification and glycation of LDL in a cohort of patients with PCOS (based on NIH criteria) and BMI-matched controls. In addition to decreased cholesterol efflux capacity, we found impairment of HDL's anti-oxidant and anti-inflammatory function among patients with increased insulin resistance.

In our study, although the difference in cholesterol efflux capacity was not different between the overall PCOS and control cohort, the sub-group who are most insulin resistant showed significantly lower cholesterol efflux capacity. Cholesterol efflux capacity was not decreased in patients diagnosed with PCOS based on the NIH criteria. Our findings substantiate a previous study where decreased cholesterol efflux capacity was shown within a PCOS cohort of higher BMI and prevalence of metabolic syndrome.[23] As we have also observed a negative association between body weight and cholesterol efflux capacity, the differences in BMI is likely to also have contributed to the differences in cholesterol efflux capacity. PON1 activity was lower in PCOS independent of BMI, and along with higher levels of SAA with increasing insulin resistance, implicates diminished anti-oxidative and anti-inflammatory function. SAA impairs both the cholesterol efflux capacity as well as the anti-inflammatory properties of HDL.[38] Its production is primarily hepatic and this is increased markedly in inflammation.[39] The overall picture is therefore one of general impairment in HDL function as insulin resistance increases. Indeed, patients with HOMA-IR in the lower tertile did not show evidence of impairment in HDL function despite the presence of PCOS, highlighting the central role of insulin resistance.

In conjunction with impairment of HDL functionality, we have also demonstrated an increase in markers of atherogenic modification of LDL, again predominantly within the cohort which is most insulin resistant. A tendency towards the highly atherogenic sdLDL in PCOS has been previously established.[10] Here, we have shown this to be predominantly among patients with increased insulin resistance, and when compared to a healthy population matched for BMI, the PCOS patients with low level insulin resistance do not demonstrate this shift in LDL particle size. The shift towards sdLDL reflects the metabolic state of insulin resistance where the associated increase in triglycerides results in a shift towards triglyceride-rich lipoproteins which

remains longer within the circulation, and through lipolysis results in generation of the smaller and denser particles.[40] sdLDL is highly susceptible to atherogenic modification, both oxidation and glycation, compared to the larger buoyant LDL subfractions.[12-14, 18] This susceptibility relates to the longer duration within circulation compared to the larger LDL particles which are removed more readily via LDL receptors, and added to this, sdLDL have also been shown to have reduced anti-oxidant content[14] and significant depletion of free cholesterol, cholesteryl esters, and phospholipids that results in susceptibility to oxidative modification.[13] Oxidative modification of LDL triggers a cascade of immune and inflammatory responses, with generation of pro-atherogenic and pro-inflammatory oxidation-specific epitopes. Consistent with high levels of OxLDL, we also found higher levels of LpPLA2 predominantly within the upper tertile of insulin resistance. LpPLA2 is an emerging biomarker of CVD which circulates bound to LDL and, to a lesser proportion, to HDL particles. Its activity is closely related to oxidative modification of LDL and atherosclerotic plaque development.[41] LpPLA2 is upregulated in rupture-prone atheromas and hydrolyses OxPL on LDL leading to production of pro-atherogenic lysophosphatidylcholine and oxidised non-esterified fatty acids. The increased LpPLA2 would also substantiate the impairment of HDL function with insulin resistance. Approximately 20% of LpPLA2 is bound to HDL, which reverses its effect from pro-atherogenic effect to anti-atherogenic,[42, 43] and improvement in glycaemic profile in type 2 diabetes was shown to reduce total LpPLA2 and redistribute LpPLA2 to a higher fraction on HDL.[41] Furthermore, reduction in PON1 activity and increase in LpPLA2 could also impair HDL's anti-inflammatory function, potentiating inflammation and insulin resistance.[19]

Interestingly, despite higher levels of both OxLDL and LpPLA2 with increasing insulin resistance, similar observation was not made with OxPL-apoB and OxPL-apo(a). Importantly, Lp(a) levels are uniform across the spectrum of insulin resistance and among the control group. Baseline serum Lp(a) levels predominantly genetically predetermined and are inversely related to the number of kringle IV type 2 repeat sequences and therefore the molecular mass of the apo(a) component.[44] Given that OxPL is bound preferentially to Lp(a) and measurements of OxPL-apoB and OxPL-apo(a) both reflects predominantly OxPL carried on Lp(a), it is therefore expected that their levels would be highly dependent on Lp(a). This is supported by the strong

positive correlations between OxPL-apoB, OxPL-apo(a), and Lp(a) both among patients with and without PCOS. Although Lp(a) levels have been associated with insulin resistance in some studies[45, 46] and we have not observed this within our cohort. We did, however, observe a negative correlation with fasting glucose. It is possible that despite increased in generation of OxPL, OxPL measured on apoB and apo(a) are not increased due to the lower Lp(a) concentration among certain insulin-resistant patients with higher fasting glucose. We, however, do acknowledge potential limitations relating to the OxLDL assay, which is based on murine monoclonal antibody 4E6, where competition for binding with unoxidised LDL can occur at higher concentrations, with both oxidised and unoxidised LDL potentially being detected.[47] Nevertheless, both LDL-C and apoB levels within our cohort where not markedly elevated and LDL-C levels did not differ between the upper tertile of insulin resistance and controls and the concerns surrounding cross-reactivity of the assay should therefore be less of a concern here.

In addition to oxidative modification, we have also demonstrated a presence of apoB glycation in PCOS independent of BMI, and this increases with increasing insulin resistance. We have demonstrated previously that sdLDL are also more susceptible to glycation compared to large buoyant LDL.[18] Glycation of LDL can compound the process of atherogenic modification by increasing susceptibility to oxidative modification,[48] and presence of sdLDL has been proposed as a major determinant of apoB glycation,[49] adding to the body of evidence for a pro-athergenic lipoprotein profile in PCOS and insulin resistance.

We also explored the prevalence of anti-apoA-I IgG positivity among our cohort. However, despite the decreased functionality suggesting potential underlying modification of HDL which may then trigger formation of immunogenic epitopes, we have not observed an increase in prevalence of anti-apoA-I IgG positivity or increase in autoantibody titre across severity of insulin resistance or compared to controls. Anti-apoA-I IgG levels and positivity was also not associated with cholesterol efflux capacity or anti-oxidant function of HDL. Interestingly, among the overall cohort with PCOS, higher levels of MPO and IgG MDA-LDL and apoB-IC were observed with anti-apoA-I positivity, suggesting a link between anti-apoA-I IgG with inflammation and oxidation. IgG MDA-LDL and apoB-IC levels correlate with CVD risk and tend to

reflect antigen exposure following oxidative modification of LDL.[50] Conversely, IgM MDA-LDL and apoB-IC, which was similar across anti-apoA-I IgG status in our cohort, often reflect natural antibodies present from birth and may potentially have protective and atherosclerosis-modulating effects.[37] Importantly, both IgG and IgM MDA-LDL and apoB-IC alter significantly by age and ethnicity, which may also explain the lack of detectable differences when comparing between high and low insulin resistance groups and controls, which is in contrast with OxLDL and LpPLA2.

We acknowledge certain limitations in our study. Firstly, the observational design of our study restricts us from drawing strong conclusions on the cause and effect of PCOS and insulin resistance on the markers of HDL functionality and lipoprotein modification. Although matched for BMI, our cohort with PCOS is younger, and this may have an impact on the variables compared, especially the indirect biomarkers of LDL oxidation. As discussed above, there are limitations relating to the assay used for measuring OxLDL and this should be given consideration when interpreting the findings. Lastly, whilst there is evidence for a less-favourable lipoprotein profile, whether this is translates into adverse cardiovascular outcomes required further study with large scale longitudinal studies.

In summary, PCOS is associated with chronic low-grade systemic inflammation with a wide phenotypic spectrum.[51] Insulin resistance is closely linked with central adiposity and inflammation and this inflammatory environment is likely the underlying driver for the increased CVD risk of PCOS. Overall, our study indicates that insulin resistance is key to identifying the subgroup of patients with PCOS who are most susceptible to pro-atherogenic modification of lipoproteins. The impairment HDL function is potentially both a contributor to and a consequence of the underlying pro-atherogenic process. These findings highlight insulin resistance as an important target in CVD risk modification.

6.6 REFERENCES

1. Dunaif A. *Polycystic ovary syndrome*. Boston
St. Louis, Mo.: Blackwell Scientific Publications ;
Distributors, USA and Canada, Mosby-Year Book; 1992.

2. Rotterdam EA-SPcwg. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod.* 2004;19:41-47.

3. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. *Nat Rev Endocrinol.* 2011;7:219-231.

4. Yildiz BO, Bozdag G, Yapici Z, Esinler I, Yarali H. Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. *Hum Reprod.* 2012;27:3067-3073.

5. Wild RA, Carmina E, Diamanti-Kandarakis E, et al. Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society. *J Clin Endocrinol Metab.* 2010;95:2038-2049.

6. Moran LJ, Misso ML, Wild RA, Norman RJ. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod Update.* 2010;16:347-363.

7. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. *Diabetes.* 1989;38:1165-1174.

8. Wang ET, Calderon-Margalit R, Cedars MI, et al. Polycystic ovary syndrome and risk for long-term diabetes and dyslipidemia. *Obstet Gynecol.* 2011;117:6-13.

9. Wild RA, Rizzo M, Clifton S, Carmina E. Lipid levels in polycystic ovary syndrome: systematic review and meta-analysis. *Fertil Steril.* 2011;95:1073-1079 e1071-1011.

10. Dejager S, Pichard C, Giral P, et al. Smaller LDL particle size in women with polycystic ovary syndrome compared to controls. *Clin Endocrinol (Oxf).* 2001;54:455-462.

11. Ai M, Otokozawa S, Asztalos BF, et al. Small dense LDL cholesterol and coronary heart disease: results from the Framingham Offspring Study. *Clin Chem.* 2010;56:967-976.

12. Packard C, Caslake M, Shepherd J. The role of small, dense low density lipoprotein (LDL): a new look. *Int J Cardiol.* 2000;74 Suppl 1:S17-22.

13. Ohmura H, Mokuno H, Sawano M, et al. Lipid compositional differences of small, dense low-density lipoprotein particle influence its oxidative susceptibility: possible implication of increased risk of coronary artery disease in subjects with phenotype B. *Metabolism.* 2002;51:1081-1087.

14. Tribble DL, Rizzo M, Chait A, Lewis DM, Blanche PJ, Krauss RM. Enhanced oxidative susceptibility and reduced antioxidant content of metabolic precursors of small, dense low-density lipoproteins. *Am J Med.* 2001;110:103-110.

15. Taleb A, Witztum JL, Tsimikas S. Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark Med.* 2011;5:673-694.

16. Leibundgut G, Scipione C, Yin H, et al. Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a). *J Lipid Res.* 2013;54:2815-2830.

17. Nordestgaard BG, Langsted A. Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology. *J Lipid Res.* 2016;57:1953-1975.

18. Younis NN, Soran H, Pemberton P, Charlton-Menys V, Elseweidy MM, Durrington PN. Small dense LDL is more susceptible to glycation than more buoyant LDL in Type 2 diabetes. *Clin Sci (Lond).* 2013;124:343-349.

19. Femlak M, Gluba-Brzozka A, Cialkowska-Rysz A, Rysz J. The role and function of HDL in patients with diabetes mellitus and the related cardiovascular risk. *Lipids Health Dis.* 2017;16:207.

20. Stahlman M, Fagerberg B, Adiels M, et al. Dyslipidemia, but not hyperglycemia and insulin resistance, is associated with marked alterations in the HDL lipidome in type 2 diabetic subjects in the DIWA cohort: impact on small HDL particles. *Biochim Biophys Acta.* 2013;1831:1609-1617.

21. Mackness MI, Durrington PN, Mackness B. How high-density lipoprotein protects against the effects of lipid peroxidation. *Curr Opin Lipidol.* 2000;11:383-388.

22. Soran H, Hama S, Yadav R, Durrington PN. HDL functionality. *Curr Opin Lipidol.* 2012;23:353-366.

23. Roe A, Hillman J, Butts S, et al. Decreased cholesterol efflux capacity and atherogenic lipid profile in young women with PCOS. *J Clin Endocrinol Metab.* 2014;99:E841-847.

24. Chistiakov DA, Orekhov AN, Bobryshev YV. ApoA1 and ApoA1-specific self-antibodies in cardiovascular disease. *Lab Invest.* 2016;96:708-718.

25. Vuilleumier N, Rossier MF, Pagano S, et al. Anti-apolipoprotein A-1 IgG as an independent cardiovascular prognostic marker affecting basal heart rate in myocardial infarction. *Eur Heart J.* 2010;31:815-823.

26. Antiochos P, Marques-Vidal P, Virzi J, et al. Anti-Apolipoprotein A-1 IgG Predict All-Cause Mortality and Are Associated with Fc Receptor-Like 3 Polymorphisms. *Front Immunol.* 2017;8:437.

27. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* 1985;28:412-419.

28. Tsimikas S, Lau HK, Han KR, et al. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation.* 2004;109:3164-3170.

29. Tsimikas S, Fazio S, Viney NJ, Xia S, Witztum JL, Marcovina SM. Relationship of lipoprotein(a) molar concentrations and mass according to lipoprotein(a) thresholds and apolipoprotein(a) isoform size. *J Clin Lipidol.* 2018;12:1313-1323.

30. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol.* 2010;30:796-801.

31. Khera AV, Cuchel M, de la Llera-Moya M, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med.* 2011;364:127-135.

32. Charlton-Menys V, Liu Y, Durrington PN. Semiautomated method for determination of serum paraoxonase activity using paraoxon as substrate. *Clin Chem.* 2006;52:453-457.

33. Leibundgut G, Arai K, Orsoni A, et al. Oxidized phospholipids are present on plasminogen, affect fibrinolysis, and increase following acute myocardial infarction. *J Am Coll Cardiol.* 2012;59:1426-1437.

34. Tsimikas S, Willeit P, Willeit J, et al. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net

reclassification of cardiovascular events. *J Am Coll Cardiol.* 2012;60:2218-2229.

35. Arai K, Orsoni A, Mallat Z, et al. Acute impact of apheresis on oxidized phospholipids in patients with familial hypercholesterolemia. *J Lipid Res.* 2012;53:1670-1678.

36. Tsimikas S, Witztum JL, Miller ER, et al. High-dose atorvastatin reduces total plasma levels of oxidized phospholipids and immune complexes present on apolipoprotein B-100 in patients with acute coronary syndromes in the MIRACL trial. *Circulation.* 2004;110:1406-1412.

37. Ravandi A, Boekholdt SM, Mallat Z, et al. Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study. *J Lipid Res.* 2011;52:1829-1836.

38. Han CY, Tang C, Guevara ME, et al. Serum amyloid A impairs the antiinflammatory properties of HDL. *J Clin Invest.* 2016;126:266-281.

39. Uhlar CM, Whitehead AS. Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem.* 1999;265:501-523.

40. Packard CJ. Triacylglycerol-rich lipoproteins and the generation of small, dense low-density lipoprotein. *Biochem Soc Trans.* 2003;31:1066-1069.

41. De Stefano A, Mannucci L, Tamburi F, et al. Lp-PLA2, a new biomarker of vascular disorders in metabolic diseases. *Int J Immunopathol Pharmacol.* 2019;33:2058738419827154.

42. Stafforini DM, Carter ME, Zimmerman GA, McIntyre TM, Prescott SM. Lipoproteins alter the catalytic behavior of the platelet-activating factor acetylhydrolase in human plasma. *Proc Natl Acad Sci U S A.* 1989;86:2393-2397.

43. Theilmeier G, De Geest B, Van Veldhoven PP, et al. HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice. *FASEB J.* 2000;14:2032-2039.

44. Utermann G, Menzel HJ, Kraft HG, Duba HC, Kemmler HG, Seitz C. Lp(a) glycoprotein phenotypes. Inheritance and relation to Lp(a)-lipoprotein concentrations in plasma. *J Clin Invest.* 1987;80:458-465.

45. Rainwater DL, Haffner SM. Insulin and 2-hour glucose levels are inversely related to Lp(a) concentrations controlled for LPA genotype. *Arterioscler Thromb Vasc Biol.* 1998;18:1335-1341.

46. Marzano L, Colussi G, Del Torre M, Sechi LA, Catena C. Relationships of plasma lipoprotein(a) levels with insulin resistance in hypertensive patients. *Metabolism.* 2014;63:1439-1446.

47. Holvoet P, Donck J, Landeloos M, et al. Correlation between oxidized low density lipoproteins and von Willebrand factor in chronic renal failure. *Thromb Haemost.* 1996;76:663-669.

48. Moro E, Alessandrini P, Zambon C, et al. Is glycation of low density lipoproteins in patients with Type 2 diabetes mellitus a LDL pre-oxidative condition? *Diabet Med.* 1999;16:663-669.

49. Younis N, Charlton-Menys V, Sharma R, Soran H, Durrington PN. Glycation of LDL in non-diabetic people: Small dense LDL is preferentially glycated both in vivo and in vitro. *Atherosclerosis.* 2009;202:162-168.

50. Prasad A, Clopton P, Ayers C, et al. Relationship of Autoantibodies to MDA-LDL and ApoB-Immune Complexes to Sex, Ethnicity, Subclinical Atherosclerosis, and Cardiovascular Events. *Arterioscler Thromb Vasc Biol.* 2017;37:1213-1221.

51. Duleba AJ, Dokras A. Is PCOS an inflammatory process? *Fertil Steril.* 2012;97:7-12.

CHAPTER 7: EFFECT OF EXTENDED RELEASE NIACIN ON LIPOPROTEIN(A) AND OXIDISED PHOSPHOLIPIDS ON APOLIPOPROTEIN B-100

To be submitted

Author's contribution:

This is a completed randomised, double-blind, placebo-controlled crossover trial.

The author arranged for shipping of samples to University of California San Diego where the oxidised phospholipids and lipoprotein(a) assays were undertaken. The author undertook data interpretation and analysis. He undertook research of available literature, wrote the first draft, and produced the final draft with Dr Handrean Soran.

Effect of extended release niacin on lipoprotein(a) and oxidised phospholipids on apolipoprotein B-100

Jan Hoong Ho[1,2], Rahul Yadav[1], Safwaan Adam[1,2], Yifen Liu[1], Xiaohong Yang[3], Paul N Durrington[1], Sotirios Tsimikas[3], Handrean Soran[1,2]

[1]Lipid Research Group, The University of Manchester, Manchester, UK
[2]Cardiovascular Trials Unit, Manchester University NHS Foundation Trust, Manchester, UK
[3]Sulpizio Cardiovascular Center, Division of Cardiovascular Medicine, University of California, San Diego, USA

Corresponding author:

Dr Handrean Soran MSc, MD, FRCP
Consultant Physician and Endocrinologist
E-mail: hsoran@aol.com; handrean.soran@mft.nhs.uk

7.1 ABSTRACT

Background and aims: Previous studies have found reductions in lipoprotein(a) [Lp(a)] and oxidised phospholipids (OxPL) on apolipoprotein B-100 (OxPL-apoB) with extended release niacin (ERN) and increases with statin therapy. Our study sought to determine the effect of ERN with laropiprant (ERN/LRP) on Lp(a), OxPL-apoB, and biomarkers of LDL oxidation when added to high-intensity statin therapy in patients with low-density lipoprotein cholesterol above treatment target of 1.8 mmol/L.

Methods: Lipid and metabolic blood parameters, OxLDL, OxPL-apoB, IgG and IgM autoantibodies to MDA-LDL, IgG and IgM apoB immune complexes were measured in 27 patients, in a previously completed randomised, double-blind, placebo-controlled crossover trial with ERN/LRP and placebo. Lp(a) was measured using the Mercodia ELISA assay and an in-house ELISA assay at the research laboratory of University of California San Diego (UCSD).

Results: Lp(a)-Mercodia was lower following ERN/LRP compared to placebo [11.6 (2.9–43.1) vs 16.7 (5.9–55.0) mg/dl, $P<0.001$], but did not achieve statistical significance on the UCSD assay [3.5 (1.9–21.9) vs 5.0 (1.9–27.0) mg/dl, $P=0.280$]. OxPL-apoB did not differ significantly between the two treatment groups [6.1 (4.0–7.3) vs 5.2 (4.1–9.3) nM, $P=0.596$]. OxLDL was lower following ERN/LRP [16.7 (9.3–20.6) vs 18.4 (11.9–22.6) ng/ml, $P=0.010$] and was strongly correlated with LDL-C (Spearman's $\rho=0.746$, $P<0.001$), and apoB (Spearman's $\rho=0.737$, $P<0.001$). There were no significant differences in IgG and IgM autoantibodies to MDA-LDL and IgG and IgM apoB immune complexes.

Conclusion: ERN/LRP results in lower Lp(a) levels but not OxPL-apoB when added to high-intensity statin therapy. The difference in Lp(a) measurements between assays highlights the need for standardisation of methods.

KEYWORDS

Extended-release niacin, laropiprant, lipoprotein(a), oxidised phospholipids, oxidised LDL, autoantibodies to oxidised LDL, apoB immune complexes.

7.2 INTRODUCTION

Oxidative stress and inflammation are well established as key processes in atherosclerotic cardiovascular disease.[1, 2] Oxidative modification of low-density lipoprotein (LDL) results in generation of oxidation specific epitopes such as oxidised phospholipids (OxPL) and malondialdehyde epitopes.[3] OxPLs are highly prevalent in atherosclerotic lesions and are potent mediators of inflammation and plaque destabilisation.[4]

Lipoprotein(a) [Lp(a)], an independent and causal risk factor for cardiovascular disease,[5-7] is complex lipoprotein composed of apolipoprotein(a) [apo(a)] covalently linked via a disulphide bond to a single molecule of apolipoprotein B-100 (apoB).[8] Lp(a) is the preferential lipoprotein carrier for OxPL and is the main contributor of OxPL measured on apolipoprotein B-100 containing lipoproteins (OxPL-apoB), which in turn reflects the Lp(a)-mediated effects in cardiovascular disease. High levels of OxPL-apoB are associated with coronary,[9, 10] carotid,[11, 12] and peripheral[11, 13] atherosclerotic cardiovascular disease, progression of calcific aortic stenosis,[14-16] and cardiac-related mortality.[17, 18] Measurements of Lp(a) are generally reported in mass as mg/dl or apo(a) particle concentration in nmol/L. Huge variations in the molecular weight and carbohydrate content of the apo(a) component, and similarly the cholesterol and phospholipid content of the apoB component have contributed to the lack of standardisation of different methods for measuring Lp(a) levels.

Compared with the database for predicting cardiovascular disease risk, the body of evidence for the impact of therapeutic interventions on OxPL-apoB is less established. It has, however, been noted that OxPL-apoB levels can increase following statin therapy and low-fat diet, and up to 89% reduction can be expected following antisense oligonucleotide therapy, with similar effects also observed in Lp(a). Niacin has a broad range of lipid-modifying effects[19] which include an approximate 20% reduction in OxPL-apoB and Lp(a).[20] Extended-release niacin (ERN) have been used in combination with laropiprant (LRP) to reduce prostaglandin D2 receptor mediated flushing. We previously conducted a randomised double-blind, placebo-controlled crossover trial, and observed reductions in LDL-associated mediators of vascular inflammation including oxidised LDL (OxLDL), lipoprotein phospholipase A2 (LpPLA2), and lysophosphatidyl choline (lyso-PC), and

macrophage chemoattractant protein-1 (MCP1) following ERN/LRP.[21] Here, we extended the assessment of the anti-inflammatory and anti-oxidative effects of ERN/LRP to OxPL-apoB and biomarkers of LDL oxidation. We also compared the Lp(a) measurements using 2 different assays in this cohort.

7.3 METHODS

Study design and participants

This is a completed randomised, double-blind, placebo-controlled crossover trial with trial medication ERN/LRP and image-matched placebo supplied by Merch, Sharp & Dohme Ltd as previously described.[21] Participants aged 20-75 years with dyslipidaemia on maximal tolerated statin and/or ezetimibe but not achieving low-density lipoprotein cholesterol (LDL-C) target of less than 1.8 mmol/L (70 mg/L) were recruited into the study. Exclusion criteria included pregnancy, breast feeding, active peptic ulcer disease, renal impairment (eGFR ≤ 59), alanine aminotransferase >1.5 times upper limit of normal, current use of niacin, fibrates or Omacor, and established allergic reaction to niacin.

All patients underwent a 4-week placebo run-in period, followed by a 12-week first treatment period where patients were randomised to either ERN/LRP (1g/20mg ERN/LRP for 4 weeks following by an increase to 2g/40mg ERN/LRP for 8 weeks) or placebo. This was then followed by a 4-week placebo washout period and a second treatment period where patients initially randomised to ERN/LRP now received placebo and vice versa. Ezetimibe was discontinued on the first visit while statin dose was unchanged through the study period. Twenty-seven out of the 36 recruited patients completed the study and the patients acted as their own controls.

The primary endpoint of the trial was the increase in high-density lipoprotein cholesterol (HDL-C) in statin-treated dyslipidaemic patients following treatment with ERN/LRP compared with placebo. In this study, we undertook a post-hoc analyses

on the effect of ERN/LRP on Lp(a), oxidised phospholipids and IgG and IgM autoantibodies and immune complexes to OxLDL.

Separation of serum and plasma

Fasting blood samples were obtained from baseline, and at the end of the first and second treatment period were used for analysis. Serum and EDTA-plasma were isolated within 2 hours of collection by centrifugation at 2000 x g for 15 minutes at 4°C.

Laboratory analyses

Total cholesterol and triglycerides were measured using cholesterol oxidase phenol 4-aminoantipyrine peroxidase and glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase methods respectively. HDL-C was assayed using a second-generation homogenous direct method (Roche Diagnostics, Burgess Hill, UK), and apolipoprotein A-I (apoA-I) and apolipoprotein B-100 (apoB) using immunoturbidimetric assays. All tests were performed on a Cobas Mira analyser (Horiba ABX Diagnostics, Nottingham, UK). The laboratory participated in the Randox International Quality Assessment Scheme (Randox Laboratories, Dublin, Ireland). LDL-C was estimated using the Friedewald formula.[22]

Measurement of lipoprotein(a)

Lp(a) determined using a commercial assay from Mercodia (Mercodia, Upsala, Sweden) and an in-house immunoassay at the University of California San Diego. The Mercodia Lp(a) assay is a two-site enzyme sandwich ELISA using two monoclonal antibodies directed against separate antigenic determinants on the apolipoprotein(a) [apo(a)] molecule. The concentration of Lp(a) is measured in U/L and converted to mg/dl using a conversion factor of 1 U/L = 0.1254 mg/dl.

Measurements were then repeated using an in-house chemiluminescent ELISA method at the research laboratory University of California San Diego.[23, 24] Briefly, 5 µg/ml of murine monoclonal antibody MB47 is plated on micro-titre well plates, capturing all apoB-containing lipoprotein particles in plasma diluted to 1:400 to yield a non-saturating amount of Lp(a). Apo(a) particles linked to apoB were then detected using biotinylated murine monoclonal antibody LPA4. Free apo(a) are not detected by this assay.

OxPL-apoB, MDA-LDL autoantibody titres and apolipoprotein B-100 immune complexes

Measurement of OxPL-apoB was undertaken using chemiluminescent immunoassay as previously described.[3] This is performed using murine monoclonal antibody E06 which binds specifically to phosphocholine (PC) on oxidised and not native phospholipids. Plasma diluted to 1:50 in 1% BSA in TBS was added to microtitre wells coated with monoclonal antibody MB47 specific to apoB. A saturating amount of apoB was added, giving equal numbers of apoB particles captured in each well. OxPL-apoB content was then determined using biotinylated E06 and values reported as nanomolar (nM) PC-OxPL using a standard curve of nM PC equivalents.[13]

IgG and IgM autoantibodies to malondialdehyde-modified LDL (MDA-LDL), and IgG and IgM apoB immune complexes (apoB-IC) were determined using chemiluminescent ELISAs as previously described.[25, 26] Briefly, for IgG and IgM

MDA-LDL, 1:200 dilution of plasma was added to micro-titre wells coated with 5 µg/ml MDA-LDL. Alkaline phosphatase-labelled goat anti-human IgG and IgM were used to determine binding to MDA-LDL. For IgG and IgM apoB-IC, 1:50 dilution of plasma was added to microtiter wells coated with murine monoclonal antibody MB47 and saturating amount of apoB. Similarly, alkaline phosphatase-labelled goat anti-human IgG and IgM were used to detect binding as above.

OxLDL was determined using sandwich ELISA immunoassay kits from Mercodia (Mercodia, Upsala, Sweden).

Statistical analyses

Continuous data were presented as mean and standard deviation for parametric variables and median and interquartile range non-parametric variables. Normality of data distribution was determined using Shapiro-Wilk test and visualising of histograms. Presence of significant carryover effects were determined prior to assessment of treatment effect. Comparison of variables and treatment effects between ERN/LRP and placebo were performed using independent samples t-test for parametric distribution and Mann-Whitney U test for non-parametric distribution. Correlations between variables were assessed using Spearman's analyses. A P-value of less than 0.05 was considered to be statistically significant. Data analyses were undertaken using SPSS for Mac (Version 23.0, IBM Statistics, Armonk, New York, USA) and figures produced using GraphPad Prism for Mac (Version 8.00, GraphPad Softrware, La Jolla California, USA).

7.4 RESULTS

Baseline characteristics

Twenty-seven patients completed the study and their baseline characteristics are shown in Table 7.1. Within the study population, fifteen patients were on rosuvastatin 40mg, eleven on atorvastatin 80mg, and one on simvastatin 40mg daily.

Table 7.1. Baseline characteristics of patients.

Clinical characteristics	
Age, y	57 (10)
Female, n (%)	9 (33)
Smoker, n (%)	3 (10)
Type 2 diabetes, n (%)	3 (10)
Hypertension, n (%)	13 (48)
BMI, kg/m^2	48 (44–52)
SBP, mmHg	135 (14)
DBP, mmHg	76 (10)
Lipid profile	
Total cholesterol, mmol/L	6.1 (5.1–6.5)
Triglyceride, mmol/L	1.6 (1.1–2.4)
HDL-C, mmol/L	1.39 (1.19–1.71)
LDL-C, mmol/L	3.4 (2.9–4.3)
ApoB, g/L	1.27 (1.04–1.47)
ApoA-I, g/L	1.39 (1.29–1.51)
Markers of glycaemia	
HbA1c, mmol/mol	40 (38–44)
Fasting glucose, mmol/L	5.3 (4.8–6.9)
Fasting insulin, mmol/L	12.5 (7.3–20.0)
HOMA-IR	2.8 (1.6–5.0)
HOMA-B	130.2 (89.9–209.1)

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables.

Abbreviations: ApoA-I, apolipoprotein A-I, apoB, apolipoprotein B-100, BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycosylated haemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure.

Lipoprotein(a)

The Lp(a) measurements obtained using the Mercodia Lp(a) assay [Lp(a)-Mercodia] was higher compared to the UCSD assay [Lp(a)-UCSD) (Figure 7.1 and Table 7.2). Treatment with ERN/LRP was associated with a lower Lp(a) compared to placebo when measured on the Mercodia assay [11.6 (2.9–43.1) vs 16.7 (5.9–55.0) mg/dl, *P*<0.001], but did not achieve statistical significance on the UCSD assay [3.5 (1.9–21.9) vs 5.0 (1.9–27.0) mg/dl, *P*=0.280]. Lp(a)-Mercodia and Lp(a)-UCSD were strongly correlated (Spearman's ρ=0.915, *P*<0.001) (Table 7.3).

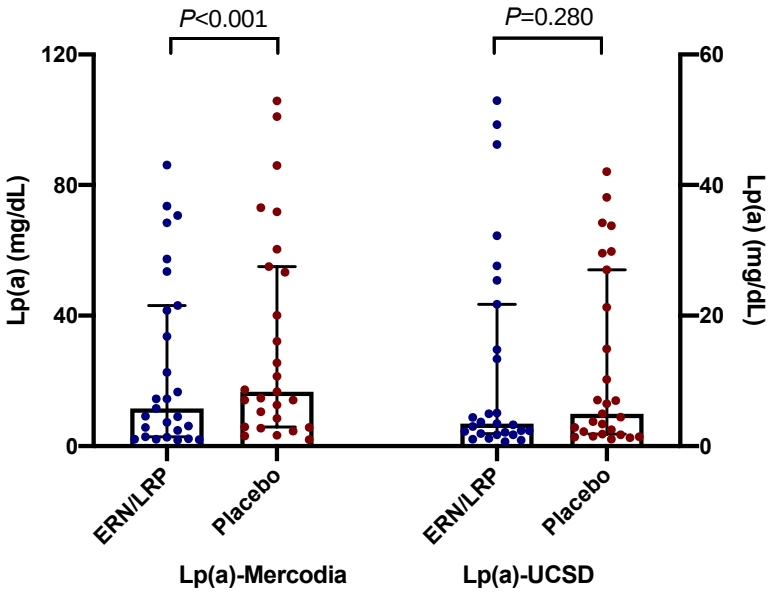


Figure 7.1. Comparison of lipoprotein(a) following treatment with ERN/LRP and placebo.

Lipoprotein(a) measurements undertaken using assay from Mercodia and in-house assay at UCSD. Median and interquartile range are shown.

Table 7.2. Lipoprotein(a) and markers of lipoprotein-associated oxidative stress following treatment with ERN/LRP and placebo.

	ERN/LRP (n=27)	**Placebo (n=27)**	***P*-value**
Lp(a)-Mercodia, mg/dL	11.6 (2.9–43.1)	16.7 (5.9–55.0)	**<0.001****
Lp(a)-UCSD, mg/dL	3.5 (1.9–21.9)	5.0 (1.9–27.0)	0.280
OxPL-apoB, nM	6.1 (4.0–7.3)	5.2 (4.1–9.3)	0.596
OxLDL, ng/mL	16.7 (9.3–20.6)	18.4 (11.9–22.6)	**0.010***
IgG MDA-LDL, RLU	14233 (9738–20737)	12484 (10115–18296)	0.259
IgM MDA-LDL, RLU	17895 (12483–29099)	16733 (10341–23842)	0.185
IgG apoB-IC, RLU	48083 (41018–71044)	45636 (41870–58111)	0.202
IgM apoB-IC, RLU	16070 (11500–22476)	15275 (9828–22156)	0.350

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables. *P*-values for the significance of differences between ERN/LRP and placebo are shown. ***P*<0.01, **P*<0.05

Abbreviations: ApoB, apolipoprotein B-100; IC, immune complex; LDL, low density lipoprotein; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidised LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; RLU, reactive light units.

Table 7.3. Bivariate Spearman correlation between lipoprotein(a), OxPL-apoB, and markers of lipoprotein-associated oxidative stress following ERN/LRP.

	Lp(a)-Mercodia	***Lp(a)-UCSD***	***OxPL-apoB***	***IgG MDA-LDL***	***IgM MDA-LDL***	***IgG apoB-IC***	***IgM apoB-IC***	***OxLDL***
Lp(a)-Mercodia, mg/dL	-							
Lp(a)-USCD, mg/dL	**0.915****	-						
OxPL-apoB, nM	**0.529****	**0.562****	-					
IgG MDA-LDL, RLU	0.074	0.033	**0.388***	-				
IgM MDA-LDL, RLU	0.153	0.183	0.376	0.297	-			
IgG apoB-IC, RLU	-0.081	-0.076	**0.351****	**0.636****	0.125	-		
IgM apoB-IC, RLU	0.160	0.221	**0.433***	0.317	**0.754****	0.230	-	
OxLDL, ng/mL	0.105	-0.029	-0.016	-0.060	-0.236	-0.089	-0.379	-

Abbreviations: ApoB, apolipoprotein B-100; IC, immune complex; Lp(a), lipoprotein(a); MDA-LDL, malondialdehyde-modified LDL; OxPL-apoB, oxidised phospholipids on apolipoprotein B-100; RLU, reactive light units.

**P*<0.05, ***P*<0.01

OxPL-apoB, and markers of lipoprotein-associated oxidative stress

OxPL-apoB did not differ significantly between the two treatment groups [6.1 (4.0–7.3) vs 5.2 (4.1–9.3) nM, *P*=0.596], while OxLDL was significantly lower following ERN/LRP compared to placebo [16.7 (9.3– 20.6) vs 18.4 (11.9–22.6) ng/mL, *P*=0.010]. There were no significant differences in IgG MDA-LDL [14233 (9738–20737) vs 12484 (10115–18296) RLU, *P*=0.259], IgM MDA-LDL [17895 (12483–29099) vs 16733 (10341–23842) RLU, *P*=0.185], IgG apoB-IC [48083 (41018–71044) vs 45636 (41870–58111) RLU, *P*=0.202] and IgM apoB-IC [16070 (11500–22476) vs 15275 (9828–22156) RLU, *P*=0.350] (Table 7.2).

Correlation between lipoprotein(a), OxPL-apoB, OxLDL, autoantibodies to MDA-LDL, and apolipoprotein B-100 immune complexes following ERN/LRP

OxPL-apoB correlated positively with both Lp(a)-Mercodia (Spearman's ρ=0.529, *P*=0.005) and Lp(a)-UCSD (Spearman's ρ=0.562, *P*=0.002) (Table 7.3). OxPL-apoB also correlated positively with IgG MDA-LDL (Spearman's ρ=0.388, *P*=0.045), IgG apoB-IC (Spearman's ρ=0.351, *P*=0.009), and IgM apoB-IC (Spearman's ρ=0.433, *P*=0.024). The correlation between OxPL-apoB and IgM MDA-LDL did not achieve statistical significance (Spearman's ρ=0.376, *P*=0.053). There was no significant correlation between OxPL-apoB and OxLDL (Spearman's ρ=-0.016, *P*=0.937). IgG MDA-LDL correlated positively with IgG apoB-IC (Spearman's ρ=0.636, *P*<0.001) and likewise, there was also a positive correlation between IgM MDA-LDL and IgM apoB-IC (Spearman's ρ=0.754, *P*<0.001).

Lipid profile and markers of glycaemia

Total cholesterol [5.1 (4.1–6.6) vs 5.7 (4.7–6.6) mmol/L, *P*=0.005], triglyceride [1.1 (0.8–1.7) vs 1.5 (1.1–2.2) mmol/L, *P*=0.010], LDL-C [2.7 (2.1–4.2) vs 3.3 (2.6–4.4) mmol/L, *P*=0.010], and apoB [0.99 (0.83–1.21) vs 1.28 (0.99–1.58) g/L, *P*<0.001] were significantly lower following treatment with ERN/LRP compared to placebo (Table 7.4). While there was a significant increase HDL-C [1.55 (1.22–1.73) vs 1.31 (1.12–1.59) mmol/L, *P*<0.001] with ERN/LRP, apoA-I [1.34 (1.22–1.51) vs 1.33 (1.21–1.48) g/L, *P*=0.750] did not differ from placebo. There was no significant difference in PCSK9 [75.7 (23.4–125.6) vs 61.0 (3.3–101.5) mmol/L, *P*=0.580] between the two treatment groups.

Correlation between lipoprotein(a), OxPL-apoB, and lipid profile after ERN/LRP

There was a negative correlation between Lp(a)-UCSD and triglyceride (Spearman's ρ=-0.386, *P*=0.047) which did not achieve statistical significance with Lp(a)-Mercodia (Spearman's ρ=-0.262, *P*=0.187) (Table 7.5). PCSK9 correlated positively with OxPL-apoB (Spearman's ρ=0.401, *P*=0.038) but not with either Lp(a)-Mercodia or Lp(a)-UCSD. Positive correlations were observed between OxLDL and total cholesterol (Spearman's ρ=0.732, *P*<0.001), LDL-C (Spearman's ρ=0.746, *P*<0.001), and apoB (Spearman's ρ=0.737, *P*<0.001).

Table 7.4. Lipid profile and markers of glycaemia following treatment with ERN/LRP and placebo.

	ERN/LRP (n=27)	Placebo (n=27)	*P*-value
Lipid profile			
Total cholesterol, mmol/L	5.1 (4.1–6.6)	5.7 (4.7–6.6)	**0.005****
Triglyceride, mmol/L	1.1 (0.8–1.7)	1.5 (1.1–2.2)	**0.012***
HDL-C, mmol/L	1.55 (1.22–1.73)	1.31 (1.12–1.59)	**<0.001****
LDL-C, mmol/L	2.7 (2.1–4.2)	3.3 (2.6–4.4)	**0.010***
ApoB, g/L	0.99 (0.83–1.21)	1.28 (0.99–1.58)	**<0.001****
ApoA-I, g/L	1.34 (1.22–1.51)	1.33 (1.21–1.48)	0.750
PCSK9, ng/mL	75.7 (23.4–125.6)	61.0 (3.3–101.5)	0.580
Markers of glycaemia			
HbA1c, mmol/mol	40 (38–44)	40 (37–42)	**0.034***
Fasting glucose, mmol/L	5.4 (4.8–6.2)	5.1 (4.8–5.8)	0.610
Fasting insulin, mmol/L	12.7 (8.2–19.8)	10.2 (6.7–21.5)	0.164
HOMA-IR	2.9 (2.2–4.9)	2.8 (1.5–5.1)	0.593
HOMA-B	140.0 (95.0–206.0)	117.3 (89.3–218.3)	0.467

Data presented as mean and standard deviation for parametric and median and interquartile range for non-parametric variables. *P*-values for the significance of differences between ERN/LRP and placebo are shown. ***P*<0.01, **P*<0.05

Abbreviations: ApoA-I, apolipoprotein A-I; apoB, apolipoprotein B-100; HbA1c, glycosylated haemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin 9.

Table 7.5. Bivariate Spearman correlation between lipoprotein(a), OxPL-apoB and lipid profile following ERN/LRP.

	Lp(a)-Mercodia	***Lp(a)-UCSD***	***OxPL-apoB***	***IgG MDA-LDL***	***IgM MDA-LDL***	***IgG apoB-IC***	***IgM apoB-IC***	***OxLDL***
Total cholesterol, mmol/L	0.035	-0.134	0.091	0.053	-0.213	-0.047	-0.206	**0.732****
Triglyceride, mmol/L	-0.262	**-0.386***	-0.301	-0.169	0.132	-0.106	0.028	0.251
HDL-C, mmol/L	-0.135	-0.204	-0.065	0.176	-0.193	-0.172	-0.040	-0.166
LDL-C, mmol/L	0.151	0.044	0.171	0.035	-0.306	0.040	-0.286	**0.746****
ApoB, g/L	0.286	0.195	0.257	0.138	-0.206	0.052	-0.198	**0.737****
PCSK9, ng/mL	0.103	0.134	**0.401***	0.147	0.192	**0.394***	0.137	0.063

Abbreviations: ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B-100; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin 9.

***P*<0.01, **P*<0.05

7.5 DISCUSSION

In this study, there was no significant change in OxPL-apoB and autoantibodies to MDA-LDL or and apoB immune complexes despite reductions in Lp(a) and OxLDL. Whilst ERN monotherapy have previously been shown to reduce OxPL-apoB,[20] this is not observed in our cohort when ERN/LRP is added to pre-existing high-intensity statin therapy. Significant increases in OxPL-apoB can be expected with statin therapy, with varying levels of increment demonstrated with different statins and populations studied. Increases of around 20 to 40% with atorvastatin 80mg,[27] and much higher increases of up to 50% in patients with clinically significant coronary artery disease have been reported.[28] Combination therapy with simvastatin, ezetimibe and niacin have resulted in a 20% mean increase in OxPL-apoB.[20] It is possible that the OxPL-apoB lowering effect of ERN may have been counteracted by the opposite effect of statin therapy although this may be offset by the statin therapy being unchanged and the crossover design of the study. Of note, higher percentage rise in OxPL-apoB compared to Lp(a) had been reported in a number of studies previously.[20, 28] The effect of dual therapy with statin and ERN has not previously been studied and future studies into the effect of different combination therapies are required.

Interestingly, Lp(a) was lower following treatment with ERN/LRP compared to placebo with both groups being on statin therapy. Again, similar to OxPL-apoB, Lp(a) levels are expected to decrease with ERN monotherapy and increase with statin therapy. In contrast to OxPL-apoB, this observation is therefore in keeping with the expected effect of ERN. ERN has been show to significantly decrease apo(a) production through direct inhibition of hepatic apo(a) gene expression.[8, 29, 30] There is also inhibition of hepatocyte diacylglycerol acyltransferase-2, leading to enhanced apoB degradation and decreased hepatic apoB-LDL and apoB-VLDL secretion, contributing to a lower Lp(a) mass.[31] Since OxPL is transported on Lp(a) attached to apo(a) and within the lipid phase, it is possible that the lowering of Lp(a) has resulted in a higher proportion of OxPL being associated with the LDL moiety and hence the lack of reduction in measured OxPL-apoB.

Lp(a) measurements in our study were undertaken using the Mercodia ELISA assay and the in-house ELISA assay at the research laboratory of UCSD. Lp(a) measured

on the Mercodia assay was significantly lower following treatment with ERN/LRP and a similar trend was observed on the UCSD assay despite not achieving statistical significance. Despite both assays being highly correlated, the Lp(a) levels measured were much higher on the Mercodia assay, further emphasising the issue and challenges surrounding Lp(a) measurement. Lp(a) mass assays determine the content of apo(a) and assumes this to be a fixed percentage of the content of the total Lp(a) mass. Since great variation exists within the contents of the LDL-like particle of Lp(a), as well as the apo(a) isoform size and content, and added to this the potential interindividual variability in proportion of Lp(a) constituents, accurate measurement of Lp(a) on mass assays can be challenging.[24] Lp(a) on the Mercodia assay is reported as U/L which is then converted to mg/dl using a fixed conversion factor. Further to the issues highlighted with mass assays, the use of a fixed conversion factor is likely not to adequately account for these variations, especially when used in interventional trials where significant lipoprotein changes occur. Within our study cohort, the differences observed between the two assays would have resulted in a different classification of cardiovascular risk attributed to Lp(a) in a proportion of patients,[32] emphasising the pressing need for standardisation of methods. Although Lp(a) assays that measure apo(a) in molar concentration overcomes the variability in mass described above, commercially available assays are not isoform independent and therefore retains its own set of issues with overestimating low values and underestimating high values.[24]

Despite significant reductions in OxLDL, autoantibodies to MDA-LDL and apoB immune complexes did not alter significantly, casting some doubts over the presence of antioxidant improvements with ERN/LRP. Furthermore, despite the increases in HDL-C, ERN/LRP have not been shown to enhance the anti-oxidant properties of HDL. Our previous study have found no improvement in paraoxonase-1 (PON1) activity and similarly another study have in fact found decreases in PON1 activity and platelet-activity factor acetylhydrolase (PAF-AH) both in plasma and on HDL.[21, 33] In fact, similar observations were made in previous statin studies, decreases in OxLDL and Lp-PLA2 (also reported in our crossover study), were not accompanied by decreases in IgG or IgM autoantibodies to MDAL-LDL or IgG or IgM apoB immune complexes.[27] Measurement of OxLDL using the Mercodia assay is based on murine monoclonal antibody 4E6 for detection of MDA-modified apoB, and similar to

our study, strong correlations with LDL-C have been reported which raises the concern for cross-reactivity.[34] Enriching plasma samples with unoxidised LDL have also previously been shown to increase OxLDL levels using this assay suggesting detection of apoB.[35] Likewise, Lp-PLA2 circulates in plasma bound to LDL and its reduction is highly associated with changes in LDL-C.[36, 37]

This study was powered for a 15% increase in HDL-C and therefore the sample size may not be sufficient for variables with a large spread of values such as Lp(a). Owing to the crossover design, there is a possibility of a carrying over effect from the first phase to second phase. This should, however, be addressed by the washout period and we have also undertaken statistical analysis for the presence of significant carryover effects prior to assessment of treatment effect. Moreover, this is unlikely given the half-life of ERN. Measurement of Lp(a) using mass assays carries the accuracy issues discussed and the use of that detect apo(a) would have provided a much more accurate reflect on Lp(a) levels. We are, however, restricted by the availability of isoform-independent assays and issues with over- and underestimation remains with commercial assays.

In conclusion, ERN/LRP results in lower Lp(a) levels but not OxPL-apoB when added to high-intensity statin therapy. Despite lower levels of OxLDL, indirect markers of LDL oxidation were not significantly different compared to placebo. The difference in Lp(a) levels reported between assays highlights the need for standardisation of method and report of Lp(a) in molar concentration using isoform independent assays.

7.6 AUTHOR CONTRIBUTIONS

R. Yadav and H. Soran conducted the initial crossover trial. J.H. Ho conducted a literature search, and conceived the idea with H. Soran. J.H. Ho, Y. Liu, and X. Yang performed laboratory analyses. J.H. Ho performed the data analyses and undertook interpretation of findings with S. Tsimikas and H. Soran. J.H. Ho produced the first draft and the final version with S. Tsimikas and H. Soran. R. Yadav, S. Adam, P.N. Durrington provided critical review for important intellectual content.

7.7 ACKNOWLEDGEMENTS

We acknowledge support from Manchester Comprehensive Local Research Network and The National Institute for Health Research/Wellcome Trust Clinical Research Facility.

7.8 DECLARATION OF INTERESTS

S. Tsimikas is a co-inventor and receives royalties from patents owned by UCSD on oxidation-specific antibodies and of biomarkers related to oxidised lipoproteins and is a co-founder and has an equity interest in Oxitope, Inc and its affiliates (“Oxitope”) as well as in Kleanthi Diagnostics, LLC (“Kleanthi”). Although these relationships have been identified for conflict of interest management based on the overall scope of the project and its potential benefit to Oxitope and Kleanthi, the research findings included in this particular publication may not necessarily relate to the interests of Oxitope and Kleanthi. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

The remaining authors have no conflict of interests to declare.

7.9 REFERENCES

1. Glass CK, Witztum JL. Atherosclerosis. the road ahead. *Cell.* 2001;104:503-516.

2. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol.* 2003;91:7A-11A.

3. Taleb A, Witztum JL, Tsimikas S. Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark Med.* 2011;5:673-694.

4. Berliner JA, Leitinger N, Tsimikas S. The role of oxidized phospholipids in atherosclerosis. *J Lipid Res.* 2009;50 Suppl:S207-212.

5. Emerging Risk Factors C, Erqou S, Kaptoge S, et al. Lipoprotein(a) concentration and the risk of coronary heart disease, stroke, and nonvascular mortality. *JAMA.* 2009;302:412-423.

6. Nordestgaard BG, Langsted A. Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology. *J Lipid Res.* 2016;57:1953-1975.

7. Durrington PN, Schofield JD, Siahmansur T, Soran H. Lipoprotein (a): gene genie. *Curr Opin Lipidol.* 2014;25:289-296.

8. Kronenberg F, Utermann G. Lipoprotein(a): resurrected by genetics. *J Intern Med.* 2013;273:6-30.

9. Kiechl S, Willeit J, Mayr M, et al. Oxidized phospholipids, lipoprotein(a), lipoprotein-associated phospholipase A2 activity, and 10-year cardiovascular outcomes: prospective results from the Bruneck study. *Arterioscler Thromb Vasc Biol.* 2007;27:1788-1795.

10. Tsimikas S, Bergmark C, Beyer RW, et al. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J Am Coll Cardiol.* 2003;41:360-370.

11. Tsimikas S, Kiechl S, Willeit J, et al. Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. *J Am Coll Cardiol.* 2006;47:2219-2228.

12. Byun YS, Yang X, Bao W, et al. Oxidized Phospholipids on Apolipoprotein B-100 and Recurrent Ischemic Events Following Stroke or Transient Ischemic Attack. *J Am Coll Cardiol.* 2017;69:147-158.

13. Bertoia ML, Pai JK, Lee JH, et al. Oxidation-specific biomarkers and risk of peripheral artery disease. *J Am Coll Cardiol.* 2013;61:2169-2179.

14. Capoulade R, Chan KL, Yeang C, et al. Oxidized Phospholipids, Lipoprotein(a), and Progression of Calcific Aortic Valve Stenosis. *J Am Coll Cardiol.* 2015;66:1236-1246.

15. Yeang C, Wilkinson MJ, Tsimikas S. Lipoprotein(a) and oxidized phospholipids in calcific aortic valve stenosis. *Curr Opin Cardiol.* 2016;31:440-450.

16. Capoulade R, Chan KL, Mathieu P, et al. Autoantibodies and immune complexes to oxidation-specific epitopes and progression of aortic stenosis: Results from the ASTRONOMER trial. *Atherosclerosis.* 2017;260:1-7.

17. Tsimikas S, Mallat Z, Talmud PJ, et al. Oxidation-specific biomarkers, lipoprotein(a), and risk of fatal and nonfatal coronary events. *J Am Coll Cardiol.* 2010;56:946-955.

18. Tsimikas S, Willeit P, Willeit J, et al. Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. *J Am Coll Cardiol.* 2012;60:2218-2229.

19. Yadav R, France M, Younis N, et al. Extended-release niacin with laropiprant : a review on efficacy, clinical effectiveness and safety. *Expert Opin Pharmacother.* 2012;13:1345-1362.

20. Yeang C, Hung MY, Byun YS, et al. Effect of therapeutic interventions on oxidized phospholipids on apolipoprotein B100 and lipoprotein(a). *J Clin Lipidol.* 2016;10:594-603.

21. Yadav R, Liu Y, Kwok S, et al. Effect of Extended-Release Niacin on High-Density Lipoprotein (HDL) Functionality, Lipoprotein Metabolism, and Mediators of Vascular Inflammation in Statin-Treated Patients. *J Am Heart Assoc.* 2015;4:e001508.

22. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem.* 1972;18:499-502.

23. Tsimikas S, Lau HK, Han KR, et al. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation.* 2004;109:3164-3170.

24. Tsimikas S, Fazio S, Viney NJ, Xia S, Witztum JL, Marcovina SM. Relationship of lipoprotein(a) molar concentrations and mass according to lipoprotein(a) thresholds and apolipoprotein(a) isoform size. *J Clin Lipidol.* 2018;12:1313-1323.

25. Tsimikas S, Witztum JL, Miller ER, et al. High-dose atorvastatin reduces total plasma levels of oxidized phospholipids and immune complexes present on apolipoprotein B-100 in patients with acute coronary syndromes in the MIRACL trial. *Circulation.* 2004;110:1406-1412.

26. Ravandi A, Boekholdt SM, Mallat Z, et al. Relationship of IgG and IgM autoantibodies and immune complexes to oxidized LDL with markers of oxidation and inflammation and cardiovascular events: results from the EPIC-Norfolk Study. *J Lipid Res.* 2011;52:1829-1836.

27. Ky B, Burke A, Tsimikas S, et al. The influence of pravastatin and atorvastatin on markers of oxidative stress in hypercholesterolemic humans. *J Am Coll Cardiol.* 2008;51:1653-1662.

28. Choi SH, Chae A, Miller E, et al. Relationship between biomarkers of oxidized low-density lipoprotein, statin therapy, quantitative coronary angiography, and atheroma: volume observations from the REVERSAL (Reversal of Atherosclerosis with Aggressive Lipid Lowering) study. *J Am Coll Cardiol.* 2008;52:24-32.

29. Croyal M, Ouguerram K, Passard M, et al. Effects of Extended-Release Nicotinic Acid on Apolipoprotein (a) Kinetics in Hypertriglyceridemic Patients. *Arterioscler Thromb Vasc Biol.* 2015;35:2042-2047.

30. Chennamsetty I, Kostner KM, Claudel T, et al. Nicotinic acid inhibits hepatic APOA gene expression: studies in humans and in transgenic mice. *J Lipid Res.* 2012;53:2405-2412.

31. Kamanna VS, Kashyap ML. Mechanism of action of niacin. *Am J Cardiol.* 2008;101:20B-26B.

32. Cegla J, Neely RDG, France M, et al. Corrigendum to "HEART UK consensus statement on Lipoprotein(a): A call to action" [Atherosclerosis 291 (2019) 62-70]. *Atherosclerosis.* 2020;296:48.

33. Masana L, Cabre A, Heras M, et al. Remarkable quantitative and qualitative differences in HDL after niacin or fenofibrate therapy in type 2 diabetic patients. *Atherosclerosis.* 2015;238:213-219.

34. Holvoet P, Harris TB, Tracy RP, et al. Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition study. *Arterioscler Thromb Vasc Biol.* 2003;23:1444-1448.

35. Tsouli SG, Kiortsis DN, Lourida ES, et al. Autoantibody titers against OxLDL are correlated with Achilles tendon thickness in patients with familial hypercholesterolemia. *J Lipid Res.* 2006;47:2208-2214.

36. Tsimikas S, Tsironis LD, Tselepis AD. New insights into the role of lipoprotein(a)-associated lipoprotein-associated phospholipase A2 in

atherosclerosis and cardiovascular disease. *Arterioscler Thromb Vasc Biol.* 2007;27:2094-2099.

37. Schaefer EJ, McNamara JR, Asztalos BF, et al. Effects of atorvastatin versus other statins on fasting and postprandial C-reactive protein and lipoprotein-associated phospholipase A2 in patients with coronary heart disease versus control subjects. *Am J Cardiol.* 2005;95:1025-1032.

CHAPTER 8: DISCUSSION

10.1 INTRODUCTION

Inflammation and oxidative stress are key contributors to the pathophysiology of CVD.[1, 2] Inflammation and oxidative stress results in atherogenic modification of LDL[3] and a shift towards dysfunctional HDL.[4] Biomarkers of LDL modification and HDL functionality are therefore of great value both in CVD risk estimation, as well as providing insights into the pathological processes underlying CVD and the effect of various therapies.

Among CVD risk markers, OxPL-apoB and Lp(a) have gained importance at the turn of the decade as independent predictors of CVD.[5-9] Although Lp(a) levels are predominantly genetically predetermined, alterations have been observed following lipid-modifying therapies such as statins,[10] niacin,[11] and antisense oligonucleotides,[12] as well as in advanced liver disease where hepatocyte synthetic activity is impaired.[13] Whether and how these changes impact on CVD outcomes remain to be established. OxPL is generated from oxidative modification of LDL.[14] It is preferentially bound to Lp(a),[7] and is thought to reflect the CVD effects of Lp(a).[14] OxPL-apoB levels are closely associated with that of Lp(a) in cross-sectional studies,[15-17] although in theory, should also be impacted by oxidative stress and LDL oxidation which is what drives the generation of OxPL.

It is clear that oxidative modification of LDL is a crucial aspect of atherogenesis and is responsible for various processes such as inflammatory activation, recruitment of immune cells, adhesion molecule and growth factor expression, and endothelial cell apoptosis, culminating in atheroma progression and plaque instability.[3] In addition to OxPL, other oxidation-specific epitopes are also generated from oxidation of LDL, and antibodies and immune complexes directed at these epitopes can be used as indirect measures of OxLDL and as biomarkers of CVD.[14]

Besides oxidative modification, LDL particles can also undergo glycation which increases its atherogenicity.[18] Glycated LDL remains in circulation for longer and is therefore more likely to participate in the atherogenic process.[19] sdLDL are more susceptible to glycation[20] and are more prevalent in obesity and metabolic syndrome.[21, 22]

While epidemiological studies have shown an inverse relationship between HDL-C and CVD risk,[23, 24] pharmacologically raising HDL-C levels have not translated into CVD outcome benefits.[25-28] This has led to a switch in focus from static measurements of HDL-C levels to the functionality of HDL. In addition to the more established markers of HDL function such as cholesterol efflux capacity and PON1 activity, the finding that specific miRNAs are transported by HDL have led to the emergence of the role of HDL-associated miRNA in HDL functionality.[29, 30] While HDL exerts various atheroprotective functions, it can be modified and become dysfunctional within an inflammatory environment,[4] and its function can therefore be affected in various metabolic conditions.

Obesity and metabolic syndrome are associated with increased CVD risk, sharing many of the key underlying disease processes with atherosclerosis.[31, 32] Insulin resistance is an important underlying factor and is closely linked to the inflammation and oxidative stress.[31] Bariatric surgery results in marked improvements in traditional CVD risk factors and significant metabolic improvements, with decrease in markers of inflammation, oxidative stress, and insulin resistance.[33-35] The underlying mechanisms driving these improvements, however, are yet to be fully established, and further understanding of the impact of obesity on these novel CVD biomarkers and its reversibility following bariatric surgery is required.

Polycystic ovarian syndrome has many of the features of metabolic syndrome and is also characterised by increased insulin resistance.[36] The prevalence of CVD risk factors is increased[37, 38] with various studies demonstrating association with an atherogenic lipoprotein profile. [39-41] Owing to its wide phenotypic spectrum and being a disease of reproductive age, PCOS represents a unique cohort for investigating the impact of insulin resistance on CVD risk markers in early cardiometabolic disease.

10.2 BARIATRIC SURGERY AND PLASMA LEVELS OF OXIDISED PHOSPHOLIPIDS AND LIPOPROTEIN(A) (CHAPTER 4)

Up until now, Lp(a), being the main lipoprotein carrier of OxPL,[7] have been closely associated with OxPL-apoB in cross-sectional population studies.[15-17] As most of the measured OxPL on the OxPL-apoB assay are on Lp(a), OxPL-apoB levels are thought to reflect the biological effects of Lp(a) on CVD.[14] Although both OxPL-apoB and Lp(a) are independent risk factors of CVD, OxPL-apoB often remains an independent predictor after adjusting for Lp(a) levels, suggesting that it provides a broader reflection of CVD risk.[8, 9]

In this study, we have demonstrated a divergence in OxPL-apoB and Lp(a) after bariatric surgery with the increase in Lp(a) levels and decrease in OxPL-apoB levels. OxPL-apoB to Lp(a) ratio is reduced as a result, indicating a reduction of OxPL carried on Lp(a). This suggests that the process of Lp(a) secretion and generation of OxPL are probably not unified in all instances. Plasma levels of Lp(a) are mostly mediated by the hepatic synthesis and it is postulated that the increase in Lp(a) is due to the improved hepatic steatosis and function following bariatric surgery. The rise in Lp(a) could also be contributed by the post-surgical reduction in triglyceride and insulin resistance, firstly a reduction in hepatic clearance of apo(a) in triglyceride-rich particles; and secondly, a reduction in insulin-mediated suppression of hepatic apo(a) synthesis.

We demonstrated significant decrease in OxPL-apoB levels in keeping with an overall reduction in oxidative stress following bariatric surgery which is supported by significant decrease in all indirect biomarkers of OxLDL. This suggests a reduction in oxidative modification of LDL and therefore less generation of OxPL. In keeping with previous studies showing accumulation of OxPL in non-alcoholic steatohepatitis,[42, 43] the reduction in OxPL-apoB with simultaneous reduction in hepatic steatosis also support the role of OxPL in the pathogenesis of hepatic steatosis.

This study also highlights the issue of OxLDL measurement, with concerns of cross-reactivity with apoB and unoxidised LDL. This is particularly relevant in interventional studies where marked changes in LDL and apoB are expected such as in bariatric surgery.

Overall, this study adds to the current evidence base for reduction in oxidative stress following bariatric surgery, which appears to be independent of surgical procedures and presence of type 2 diabetes. The rise in Lp(a) associated with metabolic improvements is unexpected and whether it translates to increased CVD risk needs to be determined, as are the impact of reduction in biomarkers of LDL oxidation on hard CVD endpoints.

10.3 ROUX-EN-Y GASTRIC BYPASS AND HDL-ASSOCIATED MIRNA (CHAPTER 5)

MiRNA are important regulators of various physiological and metabolic processes, and in recent years, have emerged as biomarkers and potential therapeutic targets for wide range of diseases, with alterations in miRNA signature being described in CVD and obesity.[44-48] More recently, HDL has been shown to transport functional miRNA and its anti-inflammatory function linked to delivery of miRNA to target cells.[29, 30, 49]

Improvements in HDL structure and function have previously been reported following bariatric surgery, although there were inconsistencies surrounding improvement in cholesterol efflux capacity. In this study, we demonstrated, for the first time, increases in HDL-associated miRNAs following RYGB in association with other markers of HDL function including cholesterol efflux capacity and PON1 activity. We therefore postulate that the enhanced expression of HDL-associated miRNAs reflects improvements in important aspects of HDL function.

Specifically, we demonstrated increased expression of miR-223, which is known to regulate inflammatory and immune processes, following RYGB. The change in HDL-associated miR-223 was inversely associated with ICAM-1, consistent with a previous study demonstrating the suppression of ICAM-1 expression in endothelial cells through transfer of HDL-associated miR-223.[30] Positive correlations between HDL-associated miR-223 and cholesterol efflux capacity an PON1 activity also add support to the overall improvement in HDL functionality.

MiR-222 is closely linked to glucose metabolisms[45] and elevated levels of miR-222 have been described in obesity and diabetes.[45, 46, 50] Following RYGB, expression of HDL-associated miRNA was increased in association with increase in HOMA-B, which is in keeping with a previous study showing glucose lowering effect of miR-222 via pancreatic bell cell proliferation in murine models.[51] We therefore hypothesise that the elevated baseline levels of miR-222 observed in obesity and diabetes may reflect a compensatory mechanism of the underlying glycaemic derangement. Interestingly, however, baseline levels of miR-222 are higher in obesity and whether this represents a compensatory mechanism in obesity for underlying metabolic derangement and if the increased expression after surgery reverts to “healthy” levels after the metabolic correction needs to be explored.

Likewise, HDL-associated miR-24 also increased after bariatric surgery which could be in response to glycaemic improvement and improvement in HDL function; whereas HDL-associated miR-126, which is expressed mainly in endothelial cells, was not significantly altered.

Overall, there is increased expression of HDL-associated miRNA following bariatric surgery, adding to the picture of improved HDL functionality reflected by cholesterol efflux capacity and PON1 activity, which may explain some of the cardiometabolic benefits of RYGB.

10.4 POLYCYSTIC OVARIAN SYNDROME AND MARKERS OF HDL FUNCTIONALITY AND LDL MODIFICATION (CHAPTER 6)

PCOS is associated with increased prevalence of CVD risk factors including an atherogenic lipid profile and impairment in cholesterol efflux capacity being demonstrated in previous studies.[39, 40, 52] PCOS has a wide phenotypic spectrum where huge variations in BMI and insulin resistance exist. Previous studies on cholesterol efflux capacity have demonstrated impairment relating to differences in BMI.[52]

In this study, markers of HDL functionality and markers of atherogenic modification of LDL were compared between PCOS and BMI-matched controls. Impairment in HDL functionality was observed reflected by both cholesterol efflux capacity, PON1 activity, and SAA relating to severity of insulin resistance. Simultaneously, there were also increase in OxLDL and LpPLA2 suggesting increase in oxidative modification of LDL, again related to severity of insulin resistance. Interestingly, the lower tertile of HOMA-IR in PCOS did not show evidence of impaired HDL functionality or LDL oxidation, suggesting that most of the atherogenic tendencies observed are likely driven by insulin resistance. There is, however, a tendency for glycation of apoB in PCOS even when BMI is matched for, which is closely linked with markers of glycaemia.

Somewhat similar to the observation made in the bariatric surgery study, OxLDL did not correlated well with OxPL-apoB. OxPL-apoB retains a strong positive correlation with Lp(a) in our cohort consistent with previous cross-sectional studies. In this cohort, although the same issue of potential cross-reactivity with the OxLDL assay exists, it is less crucial due to the lack of difference in LDL-C levels and between the groups compared. The lack of differences in OxPL-apoB and OxPL-apo(a) reflect its close relationship with Lp(a) in baseline levels, which similarly did not differ between PCOS and controls.

In addition to measures of HDL functionality, anti-apoA-I IgG levels were also compared in our cohort. Anti-apoA-I IgG has in the recent years emerged as an independent risk factor for CVD.[53, 54] The hypothesis was that modification of HDL may result in generation of these proatherogenic antibodies[55] or alternatively, the presence of anti-apoA-I IgG may negatively affect HDL function. Despite differences in measures of HDL function between groups with high and low insulin resistance, the levels and positivity of anti-apoA-I did not differ and likewise, measures of HDL function were similar between anti-apoA-I positive and negative groups. A finding of interest is the higher levels of MPO and IgG MDA-LDL and IgG apoB-IC among anti-apoA-I IgG positive patients, perhaps suggesting a link with inflammation and oxidation, although this was not supported by the other biomarkers of oxidation including OxLDL and LpPLA2.

Overall, there is evidence for impaired HDL functionality and atherogenic modification of LDL among a subgroup of patients with PCOS, with insulin resistance and BMI being key factors in identifying higher risk patients and tailoring CVD risk modification strategies.

10.5 EXTENDED RELEASE NIACIN AND PLASMA LEVELS OF LIPOPROTEIN(A) AND OXIDISED PHOSPHOLIPIDS (CHAPTER 7)

Various lipid-modifying therapies have been shown to alter levels of Lp(a) and OxPL-apoB, with increases being noted with statin therapy and decreases with niacin and the new antisense oligonucleotide therapy.[10-12] In the randomised, placebo controlled, crossover study, the impact of ERN/LRP added to maximal tolerated statin therapy on Lp(a) and OxPL-apoB levels was assessed. Lp(a) measurements were also compared between two different mass assays.

Lower levels of Lp(a) were observed following ERN/LRP add-on therapy whereas OxPL-apoB levels did not differ. The reduction in Lp(a) was expected given the Lp(a) lowering effects with niacin monotherapy previously and niacin have been shown to inhibit apo(a) production,[8] as well as enhancing apoB degradation resulting in lower Lp(a) mass.[56] Of interest is the difference in Lp(a) results generated using the two different mass assays, highlighting the issues surrounding standardisation of methods and a need for moving towards isoform independent molar methods. A major issue with mass assays is the variation in contents of the LDL-like particle of Lp(a), as well as the apo(a) isoform, both of which are not accounted for and it is assumed that each component makes up a fixed percentage of the total Lp(a) mass. This is of more importance when used to compare effect of interventions as there are likely to be lipoprotein changes and therefore alterations in contents of the lipid phase. The use of a fixed conversion factor, be it as used in the Mercodia assay, or when converting to nmol/l as reported in molar assays, adds a further layer of assumption and inaccuracy. In contrast with Lp(a), OxPL-apoB levels did not differ between ERN/LRP and placebo. This may reflect the potential differing impact of statin on Lp(a) and OxPL-apoB levels. The lowering of Lp(a) could also have therefore led to a higher number of OxPL being transported attached to the LDL

moiety, hence the OxPL-apoB measured. Also, the effects of ERN/LRP and statin on Lp(a) and OxPL-apoB when used in combination may not be the same as if extrapolated from its individual effects.

Overall, added to existing evidence, this study further confirms the Lp(a) decreasing effects of ERN/LRP. The significance of niacin-induced Lp(a) reduction on CVD outcomes needs to be established. The difference in Lp(a) values reported from different assays highlights the issues with standardisation of Lp(a) methods and the need for widespread use of isoform-independent molar assays.

10.6 LIMITATIONS

There were some important limitations in the studies presented in this thesis.

For the bariatric surgery and OxPL study (chapter 4), as it was not possible to create a true control group, we recruited patients who underwent medical weight management as a comparator group. However, due to the observational design of the study, we have no control over the choice of intervention offered to patients. The tendency for patients with higher BMI and less co-morbidities to be offered surgery with less delays is reflected in the differences in baseline characteristics between the two groups. We acknowledge that differences in age and presence of diabetes may confound our findings. However, the glycaemic control was comparable in both groups. Also, the data analyses conducted included multiple comparisons giving rise to the issue of multiple testing. It is acknowledged that beyond the primary outcome measure of OxPL-apoB and Lp(a), the sub-group analyses conducted were exploratory in nature and are limited by smaller sample size. They do, however, provide observations of interest that may offer some mechanistic explanation for the post-surgical changes observed, and serves as a basis for future studies.

Similarly, in the bariatric surgery and miRNA study (chapter 5), the issue with a true control group applies. In this study, healthy participants were recruited instead and no follow-up assessments were undertaken. The study was again observational in design and is limited by the small sample size particularly within the healthy group. As only patients who underwent RYGB were included, no procedural comparisons

were made and the findings therefore may not be extended to other bariatric procedures.

Whilst bariatric surgery is generally considered as a safe procedure with established long-term cardiometabolic benefits, it is nevertheless an invasive procedure and is not without associated complications; such anastomotic leak and stenosis in early post-operative period; and gallstone disease, nutritional deficiencies, and dumping syndrome as late complications.[57] Clinical decision for weight loss surgery should therefore be personalised taking both the potential benefits and complications into consideration.

For the PCOS study (chapter 5), although the PCOS cohort and controls were well-matched for BMI, there is a small but significant difference in age, which may have an impact on the variables measured. Again, due to the observational design of this study, whilst we are able to describe associations between insulin resistance and markers of HDL functionality and LDL modification, we are restricted in drawing conclusions on the cause and effect of these risk markers. Limitations relating to assays for measurement of OxLDL are also acknowledged.

For the ERN/LRP study, although a prior power calculation was undertaken, this was based on a 15% increase in HDL-C and therefore the sample size may not be sufficient to detect differences in variables, which are applicable to both Lp(a) and OxPL-apoB. There is also a potential for crossover effect, although this should be addressed by the washout effect which is adequate considering the half-life of ERN/LRP. Statistical analysis undertaken included exclusion of significant carrying over effects prior to assessing of treatment effect. The most important limitation, however, is the use of molar assays for the measurement of Lp(a) highlighted above which may be more relevant in the context of lipid-lowering interventions.

Whilst this study has demonstrated favourable effects of ERN/LRP, the evidence that this is translated into cardiovascular outcome benefit is not established. The use of niacin previously has also been limited due to its significant side effects, particularly severe flushing, which was improved with the addition of laropiprant.[58]

10.7 FUTURE WORK

The work in this thesis has demonstrated that bariatric surgery is associated with a divergence in OxPL-apoB and Lp(a) not previously described. The reduction in OxPL-apoB is in keeping with marked reductions in overall oxidative stress. The rise in Lp(a) levels, however requires further clarification as to whether it is 1) sustained in the longer term, and 2) translated into a negative effect on CVD risk. Likewise, whether the reductions in OxPL-apoB and biomarkers of OxLDL reflect longer term CVD outcome benefits requires further study.

Similarly, the increased expression of HDL-associated miRNA observed is thought to relate to improvements in HDL functionality. In the case of miR-222, where higher expression was observed in obesity at baseline, it was speculated that this may reflect a compensatory mechanism which needs further clarification. The cause and effect are not determined in this observational study and if this is indeed a compensatory phenomenon in response to underlying metabolic derangement, the expression of miR-222 in the longer term following metabolic correction needs to be determined. Studies into the effect of HDL-mediated delivery of specific miRNAs to target cells are required to confirm the role of these miRNA in what is a complex collection of biological processes.

We have determined that insulin resistance in PCOS is a key factor associated with LDL modification and impaired HDL functionality. This provides a platform for studies into whether therapeutic strategies targeting insulin resistance in this cohort translates into CVD outcome benefits. Similarly, the longer-term CVD benefits of weight reduction therapies are required.

The changes in Lp(a) levels with ERN/LRP add to the current evidence base for the impact of various lipid-modifying therapies on Lp(a). Further study into the impact of various combination therapies in large sample sizes are required for further clarification. Also, particularly important is the increase in Lp(a) with statin therapy, whether this confers a negative effect on CVD outcomes needs to be determined.

10.8 REFERENCES

1. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation.* 2002;105:1135-1143.

2. Kattoor AJ, Pothineni NVK, Palagiri D, Mehta JL. Oxidative Stress in Atherosclerosis. *Curr Atheroscler Rep.* 2017;19:42.

3. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med.* 1989;320:915-924.

4. Van Lenten BJ, Hama SY, de Beer FC, et al. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J Clin Invest.* 1995;96:2758-2767.

5. Emerging Risk Factors C, Erqou S, Kaptoge S, et al. Lipoprotein(a) concentration and the risk of coronary heart disease, stroke, and nonvascular mortality. *JAMA.* 2009;302:412-423.

6. Nordestgaard BG, Langsted A. Lipoprotein (a) as a cause of cardiovascular disease: insights from epidemiology, genetics, and biology. *J Lipid Res.* 2016;57:1953-1975.

7. Durrington PN, Schofield JD, Siahmansur T, Soran H. Lipoprotein (a): gene genie. *Curr Opin Lipidol.* 2014;25:289-296.

8. Tsimikas S, Brilakis ES, Miller ER, et al. Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. *N Engl J Med.* 2005;353:46-57.

9. Tsimikas S, Duff GW, Berger PB, et al. Pro-inflammatory interleukin-1 genotypes potentiate the risk of coronary artery disease and cardiovascular events mediated by oxidized phospholipids and lipoprotein(a). *J Am Coll Cardiol.* 2014;63:1724-1734.

10. Tsimikas S, Gordts P, Nora C, Yeang C, Witztum JL. Statin therapy increases lipoprotein(a) levels. *Eur Heart J.* 2019.

11. Yeang C, Hung MY, Byun YS, et al. Effect of therapeutic interventions on oxidized phospholipids on apolipoprotein B100 and lipoprotein(a). *J Clin Lipidol.* 2016;10:594-603.

12. Tsimikas S, Karwatowska-Prokopczuk E, Gouni-Berthold I, et al. Lipoprotein(a) Reduction in Persons with Cardiovascular Disease. *N Engl J Med.* 2020;382:244-255.

13. Konishi K, Miyake T, Furukawa S, et al. Advanced fibrosis of non-alcoholic steatohepatitis affects the significance of lipoprotein(a) as a cardiovascular risk factor. *Atherosclerosis.* 2020;299:32-37.

14. Taleb A, Witztum JL, Tsimikas S. Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events. *Biomark Med.* 2011;5:673-694.

15. Tsimikas S, Bergmark C, Beyer RW, et al. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J Am Coll Cardiol.* 2003;41:360-370.

16. Tsimikas S, Lau HK, Han KR, et al. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation.* 2004;109:3164-3170.

17. Leibundgut G, Scipione C, Yin H, et al. Determinants of binding of oxidized phospholipids on apolipoprotein (a) and lipoprotein (a). *J Lipid Res.* 2013;54:2815-2830.

18. Younis N, Sharma R, Soran H, Charlton-Menys V, Elseweidy M, Durrington PN. Glycation as an atherogenic modification of LDL. *Curr Opin Lipidol.* 2008;19:378-384.

19. Witztum JL, Mahoney EM, Branks MJ, Fisher M, Elam R, Steinberg D. Nonenzymatic glucosylation of low-density lipoprotein alters its biologic activity. *Diabetes.* 1982;31:283-291.

20. Younis NN, Soran H, Pemberton P, Charlton-Menys V, Elseweidy MM, Durrington PN. Small dense LDL is more susceptible to glycation than more buoyant LDL in Type 2 diabetes. *Clin Sci (Lond).* 2013;124:343-349.

21. Magkos F, Mohammed BS, Mittendorfer B. Effect of obesity on the plasma lipoprotein subclass profile in normoglycemic and normolipidemic men and women. *Int J Obes (Lond).* 2008;32:1655-1664.

22. Fan J, Liu Y, Yin S, et al. Small dense LDL cholesterol is associated with metabolic syndrome traits independently of obesity and inflammation. *Nutr Metab (Lond).* 2019;16:7.

23. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med.* 1977;62:707-714.

24. Barter P, Gotto AM, LaRosa JC, et al. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. *N Engl J Med.* 2007;357:1301-1310.

25. Investigators A-H, Boden WE, Probstfield JL, et al. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. *N Engl J Med.* 2011;365:2255-2267.

26. Group HTC, Landray MJ, Haynes R, et al. Effects of extended-release niacin with laropiprant in high-risk patients. *N Engl J Med.* 2014;371:203-212.

27. Schwartz GG, Olsson AG, Abt M, et al. Effects of dalcetrapib in patients with a recent acute coronary syndrome. *N Engl J Med.* 2012;367:2089-2099.

28. Group HTRC, Bowman L, Hopewell JC, et al. Effects of Anacetrapib in Patients with Atherosclerotic Vascular Disease. *N Engl J Med.* 2017;377:1217-1227.

29. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol.* 2011;13:423-433.

30. Tabet F, Vickers KC, Cuesta Torres LF, et al. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun.* 2014;5:3292.

31. Hotamisligil GS. Inflammation and metabolic disorders. *Nature.* 2006;444:860-867.

32. Marseglia L, Manti S, D'Angelo G, et al. Oxidative stress in obesity: a critical component in human diseases. *Int J Mol Sci.* 2014;16:378-400.

33. Yadav R, Hama S, Liu Y, et al. Effect of Roux-en-Y Bariatric Surgery on Lipoproteins, Insulin Resistance, and Systemic and Vascular Inflammation in Obesity and Diabetes. *Front Immunol.* 2017;8:1512.

34. Tozzo C, Moreira EAM, de Freitas MB, da Silva AF, Portari GV, Wilhelm Filho D. Effect of RYGB on Oxidative Stress in Adults: a 6-Year Follow-up Study. *Obes Surg.* 2020;30:3301-3308.

35. da Silva VR, Moreira EA, Wilhelm-Filho D, et al. Proinflammatory and oxidative stress markers in patients submitted to Roux-en-Y gastric bypass after 1 year of follow-up. *Eur J Clin Nutr.* 2012;66:891-899.

36. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. *Diabetes.* 1989;38:1165-1174.

37. Wild RA, Carmina E, Diamanti-Kandarakis E, et al. Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society. *J Clin Endocrinol Metab.* 2010;95:2038-2049.

38. Moran LJ, Misso ML, Wild RA, Norman RJ. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod Update.* 2010;16:347-363.

39. Wild RA, Rizzo M, Clifton S, Carmina E. Lipid levels in polycystic ovary syndrome: systematic review and meta-analysis. *Fertil Steril.* 2011;95:1073-1079 e1071-1011.

40. Dejager S, Pichard C, Giral P, et al. Smaller LDL particle size in women with polycystic ovary syndrome compared to controls. *Clin Endocrinol (Oxf).* 2001;54:455-462.

41. Macut D, Panidis D, Glisic B, et al. Lipid and lipoprotein profile in women with polycystic ovary syndrome. *Can J Physiol Pharmacol.* 2008;86:199-204.

42. Sun X, Seidman JS, Zhao P, et al. Neutralization of Oxidized Phospholipids Ameliorates Non-alcoholic Steatohepatitis. *Cell Metab.* 2020;31:189-206 e188.

43. Que X, Hung MY, Yeang C, et al. Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice. *Nature.* 2018;558:301-306.

44. Schroen B, Heymans S. Small but smart--microRNAs in the centre of inflammatory processes during cardiovascular diseases, the metabolic syndrome, and ageing. *Cardiovasc Res.* 2012;93:605-613.

45. Deiuliis JA. MicroRNAs as regulators of metabolic disease: pathophysiologic significance and emerging role as biomarkers and therapeutics. *Int J Obes (Lond).* 2016;40:88-101.

46. Ortega FJ, Mercader JM, Catalan V, et al. Targeting the circulating microRNA signature of obesity. *Clin Chem.* 2013;59:781-792.

47. Hulsmans M, De Keyzer D, Holvoet P. MicroRNAs regulating oxidative stress and inflammation in relation to obesity and atherosclerosis. *FASEB J.* 2011;25:2515-2527.

48. Vickers KC, Remaley AT. MicroRNAs in atherosclerosis and lipoprotein metabolism. *Curr Opin Endocrinol Diabetes Obes.* 2010;17:150-155.

49. Vickers KC, Remaley AT. Lipid-based carriers of microRNAs and intercellular communication. *Curr Opin Lipidol.* 2012;23:91-97.

50. Ortega FJ, Mercader JM, Moreno-Navarrete JM, et al. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes that change with insulin sensitization. *Diabetes Care.* 2014;37:1375-1383.

51. Tsukita S, Yamada T, Takahashi K, et al. MicroRNAs 106b and 222 Improve Hyperglycemia in a Mouse Model of Insulin-Deficient Diabetes via Pancreatic beta-Cell Proliferation. *EBioMedicine.* 2017;15:163-172.

52. Roe A, Hillman J, Butts S, et al. Decreased cholesterol efflux capacity and atherogenic lipid profile in young women with PCOS. *J Clin Endocrinol Metab.* 2014;99:E841-847.

53. Vuilleumier N, Rossier MF, Pagano S, et al. Anti-apolipoprotein A-1 IgG as an independent cardiovascular prognostic marker affecting basal heart rate in myocardial infarction. *Eur Heart J.* 2010;31:815-823.

54. Antiochos P, Marques-Vidal P, Virzi J, et al. Anti-Apolipoprotein A-1 IgG Predict All-Cause Mortality and Are Associated with Fc Receptor-Like 3 Polymorphisms. *Front Immunol.* 2017;8:437.

55. Chistiakov DA, Orekhov AN, Bobryshev YV. ApoA1 and ApoA1-specific self-antibodies in cardiovascular disease. *Lab Invest.* 2016;96:708-718.

56. Kamanna VS, Kashyap ML. Mechanism of action of niacin. *Am J Cardiol.* 2008;101:20B-26B.

57. Lim R, Beekley A, Johnson DC, Davis KA. Early and late complications of bariatric operation. *Trauma Surg Acute Care Open.* 2018;3:e000219.

58. Yadav R, Kwok S, Ammori BJ, Issa B, Soran H. Safety and tolerability of extended-release niacin with laropiprant. *Expert Opin Drug Saf.* 2012;11:151-159.

APPENDIX

NHS
Manchester University
NHS Foundation Trust

Study Title

Obesity-related cardiovascular risk and the effect of bariatric surgery-induced weight loss: A mechanistic study investigating the role of hormones, lipoproteins, vascular endothelial factors and small nerve fibre integrity.

- You are being invited to take part in a research study to look at cardiovascular health after bariatric surgery.
- This sheet provides you with the information about the study and how it involves you.
- Before you decide, it is important for you to understand why the research is being done and what it will involve.
- Please take time to read the following information carefully before deciding on whether to take part or not.

Introduction

Obesity is a common problem in the United Kingdom and is associated with a number of significant health conditions.

Problems with cardiovascular health are common in obesity. Obesity leads to a state of low level inflammation which is thought to drive the process leading to cardiovascular disease. A number other factors have also been proposed such as disruption in blood vessel, nerve and hormone function. Bariatric surgery and weight loss has been shown to have beneficial effects on cardiovascular health although the mechanisms by which these effects occur remain to be fully established.

This study will explore the effects of weight loss surgery on sexual and cardiovascular health, looking into the role of blood vessel function, nerve function and hormone status.

What is the purpose of this study?

The purpose of this study is to first and foremost assess for improvement in inflammation affecting blood vessels following bariatric surgery and weight loss. We will also study the effect of bariatric surgery on the other factors contributing to cardiovascular health such as blood vessel function, nerve function, and hormone status. This will help us understand why obese individuals

are at risk of encountering problems with cardiovascular health and how weight loss can have an influence on blood vessel, nerve and hormone function.

With increasing evidence supporting the link between sexual and cardiovascular health, this study will also seek to determine the effects of bariatric surgery on sexual function and explore the use of a validated questionnaire for cardiovascular risk assessment.

Why have I been chosen?

You have been scheduled for bariatric surgery and are therefore suitable for the study. We hope to confirm that bariatric surgery and weight loss results in favourable changes to the factors underlying cardiovascular disease – inflammation, blood vessel function, nerve function, and hormone status.

What will I have to do if I take part?

If you are interested in taking part in this study, we will arrange for an initial appointment at the Cardiovascular Trials Unit in Central Manchester University Hospitals where we will go through the study in detail with you and to ensure that you meet the study criteria. You will then be asked to sign a consent form for the study.

Once enrolled into the study, you will need to attend the Manchester Wellcome Trust Clinical Research Facility for 3 visits.

Your first visit will be arranged before bariatric surgery and the second and third visit at 6 months and 12 months after bariatric surgery respectively.

You will be asked to fast for at least 6 hours prior to the appointment. We will review your medical and medication history, and ask you to complete a questionnaire. You will have a brief physical examination that includes measurements of height, weight, waist circumference and blood pressure. We will perform an ECG test to assess the health of your heart.

A total of 60 ml (around 12 teaspoons) of fasting blood will be taken from you. You will be asked to give a urine sample and a stool sample for analysis. We will also ask you to provide a sample of saliva so that hormone testing can be carried out which will also help study the link between weight loss, hormonal status and cardiovascular health.

You will also undergo a series of nerve function tests which consist of:

- Short questionnaire on symptoms of nerve damage such as pain and weakness.

- Physical examination including testing your ability to sense fine touch, different temperatures and vibration in your legs.
- Nerve conduction study where nerves in your legs are stimulated resulting in momentary muscle twitching. This may cause minor fleeting discomfort.
- Autonomic function testing where you will be attached to a heart monitor will performing breathing exercises.
- Corneal sensitivity is assessed with an air puff stimulus to the front of your eyes with no direct contact.
- A corneal confocal microscope (CCM) will be used to examine the number of nerves in the front part of the eye. A drop of anaesthetic is applied to numb the front of the eye. This allows a gel on the lens of the camera to touch the front of the eye for 1-2 minutes whilst we record images of the cornea.

Each visit will take approximately 120 minutes to complete.

What will happen to the samples taken?

Urine, saliva, and most of the blood samples will be analysed in the Manchester or Salford laboratories. A small volume of blood sample will be retained for future research at the end of the study. In addition, a small frozen anonymised blood sample and stool samples will be sent to international laboratories for further tests. The tests to be carried out in these laboratories are for research and not for clinical/ diagnostic purposes. Urine, stool, and saliva samples will be destroyed at the end of the study period.

What are the possible risks of taking part?

The blood samples will be taken by an experienced doctor or nurse and the only risk involved may be some bruising at the puncture site. There are no risks involved in the nerve function tests.

Are there any possible benefits?

There are no immediate benefits to you. However, the knowledge gained from this study will help us understand the effects of obesity and subsequent weight loss surgery on cardiovascular health better. The improved knowledge on changes occurring after weight loss surgery may provide a foundation for guiding future treatment strategies for obesity and sexual dysfunction.

Will I be paid for taking part in the study?

No. But your travel expenses will be reimbursed. You have the option of receiving £30 as a single payment for each visit, or you can be reimbursed at each visit on the production of taxi receipt for attending and we will arrange a taxi (paid for by the research team) for your return after your visit.

What will happen to my clinical and personal information?

All the clinical information you provide will be encoded (so that your personal details such as name and address are secure) and stored securely. This information will not be revealed to anyone other than the researchers and your GP if you wish the latter to be informed. We would ask your permission to inform your GP of any clinically relevant abnormalities identified during the study.

Do I have to take part?

No, taking part is entirely voluntary. If you do not wish to take part you do not have to give a reason and in no way will your future treatment be affected. You can also decide to withdraw from the study at any point during the study. The intention to withdraw can be communicated either verbally or in writing to the research team and this will be documented in the participant's file. In the case of withdrawal of consent, anonymised data and samples collected up to the point of withdrawal will be retained and used for purpose of this research study. Participants, however, can request specifically that data and samples are destroyed. This can again be communicated either verbally or in writing, and will be documented in the participant's file.

Complaints

If you have any concerns about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (see below). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or the Patient Advisory Liaison Service (PALS). Details can be obtained from the hospital.

What do I do now?

If you have any questions please contact:

- Dr Jan Hoong Ho, Cardiovascular Research Group, University of Manchester, Core Technology Facility (3rd floor), 46 Grafton Street, Manchester, M13 9NT). Tel: 07809669984.

  Alternatively, you can contact the doctor whose clinics you are attending:

- Dr Handrean Soran, Consultant Physician, Department of Medicine, Manchester Royal Infirmary, Oxford Road, Manchester, M13 9WL. Tel: 0161 276 4066 (Secretary).

- Mr Basil Ammori, Consultant Surgeon, Department of Surgery, Salford Royal Hospital, Stott Lane, Salford M6 8HD. Tel: 0161 789 7373.

Thank you for taking the time to read this and considering taking part in our research. Please discuss this information with your family, friends or GP, if you wish. You will have at least 24 hours to read this information leaflet. After this time, we will contact you again to see if you are still interested in taking part.

NHS
Manchester University
NHS Foundation Trust

Obesity-related cardiovascular risk and the effect of bariatric surgery-induced weight loss: A mechanistic study investigating the role of hormones, lipoproteins, vascular endothelial factors and small nerve fibre integrity.

To be completed by the patient: **Please initial the boxes**

1. I confirm that I have read and understood the patient information sheet [version 1.0. 23.11.2016] provided for the study and I have had the opportunity to ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS trust or regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records. ☐
4. I agree to serum and plasma being retained and stored as a gift to University of Manchester and used for future ethically approved research at the end of the study. ☐
5. I agree to fat biopsy samples being retained and stored as a gift to University of Manchester and used for future ethically approved research at the end of the study. ☐
6. I consent to my general practitioner being informed of my participation in the study and of any clinically relevant information. ☐
7. I agree for my anonymised blood samples to be transferred to international laboratories for research purposes. ☐
8. I give my consent to take part in the above study including:
   a. Sexual function questionnaire ☐
   b. Blood test ☐
   c. Stool, saliva and urine samples ☐

d. Nerve function tests ☐

Name…… Date of Birth……

Signature…… Date……..

To be completed by the investigator or physician or nurse taking consent:

I confirm that I have fully explained and discussed with the patient the nature and purpose of the above study.

Name…….. Position…… (e.g. Investigator)

Signature…… Date……

Signature of physician if consent was witnessed by a nurse……

North West - Greater Manchester East Research Ethics Committee
3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 020 71048008

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

02 August 2017

Dr Handrean Soran
Department of Medicine
Manchester Royal Infirmary
Oxford Road, Manchester
M13 9WL

Dear Dr Soran

Study title:	**Obesity-related cardiovascular risk and the effect of bariatric surgery-induced weight loss: A mechanistic study investigating the role of hormones, lipoproteins, vascular endothelial factors and small nerve fibre integrity.**
REC reference:	**17/NW/0357**
Protocol number:	**PET2017**
IRAS project ID:	**220085**

Thank you for your letter of 31 July 2017, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting

documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Cover Letter]		10 May 2017
GP/consultant information sheets or letters [GP Letter]	1	02 June 2017
IRAS Application Form [IRAS_Form_03062017]		03 June 2017
Non-validated questionnaire [Medical Questionnaire]	1	27 April 2017
Other [Male SFQ]	2	01 July 2007
Other [Female SFQ]		
Other [2nd Supervisor's CV]		02 June 2017
Other [GP Letter for non-PETCT Arm]	1	02 June 2017
Other		
Participant consent form [non PET]	2	17 July 2017
Participant consent form [PET]	2	17 July 2017
Participant information sheet (PIS) [non PET]	2	17 July 2017
Participant information sheet (PIS) [PET]	2	17 July 2017
Referee's report or other scientific critique report [Referee's Report]		14 December 2016
Research protocol or project proposal	2	17 July 2017
Response to Request for Further Information		31 July 2017
Summary CV for Chief Investigator (CI) [Chief Investigator CV]		
Summary CV for student [Student's CV]		06 April 2017
Summary CV for supervisor (student research) [Supervisor CV]		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments

- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

17/NW/0357	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

pp.

Mr Simon Jones
Chair

Email:nrescommittee.northwest-gmeast@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Dr Lynne Webster, Central Manchester University Hospitals NHS Foundation Trust*

NHS
Health Research Authority

Dr Handrean Soran
Department of Medicine
Manchester Royal Infirmary
Oxford Road, Manchester
M13 9WL

Email: hra.approval@nhs.net

27 September 2017

Dear Dr Soran,

Letter of HRA Approval

Study title:	**Obesity-related cardiovascular risk and the effect of bariatric surgery-induced weight loss: A mechanistic study investigating the role of hormones, lipoproteins, vascular endothelial factors and small nerve fibre integrity.**
IRAS project ID:	**220085**
Protocol number:	**PET2017**
REC reference:	**17/NW/0357**
Sponsor	**Central Manchester University Hospitals NHS Foundation Trust**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators",* issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

IRAS project ID	220085

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **220085**. Please quote this on all correspondence.

Yours sincerely

Nabeela Iqbal
Assessor

Email: hra.approval@nhs.net

Copy to: *Dr Lynne Webster, Central Manchester University Hospitals NHS Foundation Trust, Sponsor a*

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
IRAS Application Form [IRAS_Form_03062017]		03 June 2017
Letter from funder [amgen letter]		01 June 2017
Non-validated questionnaire [Medical Questionnaire]	1	27 April 2017
Other [Consent - non-PETCT Arm]	1	21 February 2017
Other [PIS - non-PETCT Arm]	1	27 April 2017
Other [HRA SOE]		
Other [PIC SOA]		
Other [Male SFQ]	2	01 July 2007
Other [Female SFQ]		
Other [2nd Supervisor's CV]		02 June 2017
Other [GP Letter for non-PETCT Arm]	1	02 June 2017
Participant consent form [non PET]	2	17 July 2017
Participant consent form [PET]	2	17 July 2017
Participant information sheet (PIS) [non PET]	2	17 July 2017
Participant information sheet (PIS) [PET]	2	17 July 2017
Referee's report or other scientific critique report [Referee's Report]		14 December 2016
Research protocol or project proposal	2	17 July 2017
Summary CV for Chief Investigator (CI) [Chief Investigator CV]		
Summary CV for student [Student's CV]		06 April 2017
Summary CV for supervisor (student research) [Supervisor CV]		

Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.

For information on how the sponsor should be working with participating NHS organisations in England, please refer to the, *participating NHS organisations*, *capacity and capability* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections in this appendix.

The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Name: Dr Lynne Webster

Tel: 01612764125

Email: lynne.webster@cmft.nhs.uk

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	Revision made to the Patient Information sheet as follows: PISs PET CT and non PETCT have been revised to be clear what will happen to the samples include destruction at the end of the study period.
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	The Statement of Activities (SOA) will act as an agreement between participating sites.
4.2	Insurance/indemnity arrangements assessed	Yes	Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional

Section	HRA Assessment Criteria	Compliant with Standards	Comments
			indemnity provided by their medical defence organisation covers the activities expected of them for this research study
4.3	Financial arrangements assessed	Yes	This study is funded by AMGEN limited. The sponsor will not provide any funding to the NHS organisation identifying potential participants.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	For the purpose of HRA assessment, applicant has provided clarification about the tissue samples to be taken and what will happen to these after the study.
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	REC FO issued on 2nd August 2017.
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Yes	ARSAC certificate has been provided for the site.

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
There are 2 site types. Salford Hospital will act as a PIC and Central Manchester University Hospital NHS Foundation will conduct all site activity The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website. If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.
Participating NHS organisations in England **will be expected to formally confirm their capacity and capability** to host this research. • The sponsor should ensure that participating NHS organisations are provided with a copy of this letter and all relevant study documentation, and work jointly with NHS organisations to arrange capacity and capability whilst the HRA assessment is ongoing. • Further detail on how capacity and capability will be confirmed by participating NHS organisations, following issue of the Letter of HRA Approval, is provided in the *Participating NHS Organisations* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections of this appendix. • The Assessing, Arranging, and Confirming document on the HRA website provides further information for the sponsor and NHS organisations on assessing, arranging and confirming capacity and capability.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A principal investigator is required for site where all site activity is to be conducted. However, LC is not required for the PIC site. GCP training is not a generic training expectation, in line with the HRA statement on training

expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
As a study undertaken by local staff, it is unlikely that letters of access or honorary research contracts will be applicable, except where local network staff employed by another Trust (or University) are involved (and then it is likely that arrangements are already in place). Where contractual arrangements are not already in place, network staff (or similar) undertaking research activities would be expected to obtain Honorary Research Contracts on the basis of a Research Passport (if university employed) or a Letter of Access on the basis of an NHS to NHS confirmation of pre-engagement checks letter (if NHS employed). Standard DBS checks and occupational health clearance would be appropriate.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
The applicant has indicated that they intend to apply for inclusion on the NIHR CRN Portfolio.