An investigation into the interrelationship between HDL particle composition and anti-atherosclerotic functions in patients with cardiometabolic disease.

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'Everything can be taken from a man but one thing: the last of the human freedoms—to choose one's attitude in any given set of circumstances, to choose one's own way.'

Victor E. Frankl (holocaust survivor)

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Statement of Original Authorship

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree of Doctor of Philosophy, and I have not obtained a degree elsewhere based on the research presented in this submitted work.

_____

Rachel Byrne

January 2022

Collaborations

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Author Contributions

Chapter 3 – The potential of the HDL proteome to predict HDL-cholesterol efflux capacity in people with and without metabolic disease.

Rachel Byrne and Dr Fiona McGillicuddy designed all experimental work. Prof. James Gibney managed the clinical recruitment along with his team of endocrinologists including Dr Mohamed Ahmed, Dr Khalid Ahmed, Dr Anne McGowan, and Dr Anjuli Gunness, who organised and completed clinical measurements and blood lipid analysis. Rachel Byrne performed the HDL function assays, HDL isolation, HDL purification and subsequent HDL proteomic sample preparation. Dr Eugene Dillion ran the mass spectrometry experiments. Dr Anna Antoniadi generated Receiver Operating Characteristic (ROC) curves. Rachel Byrne performed all other experimental and data analysis.

Chapter 4 – Investigating the impact of type 1 diabetes mellitus and type 2 diabetes mellitus on HDL function, and composition in humans.

Rachel Byrne, Prof. James Gibney, and Dr Fiona McGillicuddy designed all experimental work. Prof. James Gibney managed the clinical recruitment for Tallaght University Hospital along with his team of endocrinologists including Dr Mohamed Ahmed, Dr Khalid Ahmed, Dr Anne McGowan, Dr Anjuli Gunness, Dr Isolda Frizelle, and Dr Agnieszka Pazderska, who organised and completed clinical measurements and blood lipid analysis. Prof. Donal O'Shea and Dr Andrew Hogan managed the recruitment from St. Vincent's University Hospital. Rachel Byrne performed the HDL function assays, HDL isolation, HDL purification and subsequent HDL proteomic sample preparation. Dr Eugene Dillion ran the mass spectrometry experiments. Rachel Byrne performed all experimental and data analysis.

Chapter 5 – Sex-specific differences in HDL function and composition in people with and without metabolic disease.

Rachel Byrne and Dr Fiona McGillicuddy designed all experimental work. Prof. James Gibney managed the clinical recruitment for Tallaght University Hospital along with his team of endocrinologists including Dr Mohamed Ahmed, Dr Khalid Ahmed, Dr Anne McGowan, Dr Anjuli Gunness, Dr Isolda Frizelle, and Dr Agnieszka Pazderska, who organised and completed clinical measurements and blood lipid analysis. Prof. Donal O'Shea and Dr Andrew Hogan managed the recruitment from St. Vincent's University Hospital.

Rachel Byrne performed the HDL function assays, HDL isolation, HDL purification and subsequent HDL proteomic sample preparation. Dr Eugene Dillion ran the mass spectrometry experiments. Rachel Byrne performed all experimental and data analysis.

Publications

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* Ahmed, M.O., and Byrne, R. are co-first authors.
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Presentations and Conferences

Oral Presentations

2021 – 45th Annual Meeting of the Irish Endocrine Society, online.

Poster presentations

2019 – Inaugural UCD School of Medicine Research Symposium, UCD, Dublin, Ireland.
2019 – 42nd Annual Meeting of the European Lipoprotein Club, Tutzing, Germany.
2020 – 88th European Atherosclerosis Society (EAS) Congress, online.
2020 – 2nd UCD School of Medicine Research Symposium, UCD, Dublin, Ireland, online.
2021 – Precision Diabetes Medicine 2021, online.

Other conferences and courses attended

2018 – SCI:COM, Science Communication Conference, Dublin, Ireland.
2018 – 3U Diabetes 6th Annual Research Conference, Royal College of Surgeons Ireland.
2019 – 13th European Nutrition Conference, Dublin, Ireland.
2021 – 89th European Atherosclerosis Society (EAS) Congress, online.
2021 – 12th International Summer School on Computational Mass Spectrometry-Based Proteomics, online.

Awards

2020 – Received UCD Seed Funding to attend 88th European Atherosclerosis Society (EAS) Congress.

Memberships

2019 – present: European Atherosclerosis Society
2019 – present: The Nutrition Society

Abstract

Diabetes is one of the fastest growing global health emergencies of the 21st century. People with type 1 diabetes mellitus (T1DM) and people with type 2 diabetes mellitus (T2DM) have an increased risk of cardiovascular disease (CVD), and furthermore, women with T1DM or T2DM have an increased risk relative to men with T1DM or T2DM. High-density lipoprotein particles (HDL-P) exert numerous atheroprotective functions including the ability to promote cholesterol efflux from peripheral cells (cholesterol efflux capacity (CEC)), antioxidant and anti-inflammatory functions. HDL-CEC, the first step of the reverse cholesterol transport pathway, is widely considered the dominant mechanism underpinning the cardioprotective properties of HDL. Small HDL-P facilitate cellular efflux via the ATP-Binding Cassette Subfamily A Member 1 (ABCA1) cholesterol transporter (ABCA1-dependent efflux), while larger HDL-P mediate efflux via ATP-binding cassette subfamily G, member 1 (ABCG1) and Scavenger Receptor Class B Type 1 (SR-B1) pathways (ABCA1-independent efflux). HDL-P carry a cargo of approximately 250 different proteins known as the HDL proteome. The functions of the proteins associated with HDL align strongly with the assigned functions of the particles indicative that measuring HDL associated proteins may serve as novel biomarkers of HDL function.

In this thesis, we hypothesised that the HDL proteome is impacted in T1DM or T2DM resulting in the generation of dysfunctional HDL-P contributing to enhanced CVD risk within these populations. We further hypothesised that the HDL proteome may serve as a surrogate biomarker for HDL-CEC and sought to identify key proteins governing HDL-CEC in people with and without metabolic disease.

In Chapter 3, we set out to investigate the relationship between HDL-associated proteins and HDL-CEC, and furthermore developed novel biomarker panels and a scoring algorithm for total CEC, ABCA1-dependent CEC and ABCA1-independent CEC. Our Receiver Operating Characteristic (ROC) area under the curve (AUC) in turn was 0.859, *P*=0.0001*** for total efflux score, 0.862, *P*=0.0001*** for ABCA1-dependent efflux score and 0.781, *P*=0.0002** for ABCA1-independent efflux score, highlighting the ability of the HDL proteome to accurately predict HDL-CEC.

In Chapter 4, we investigated the impact of T1DM, T2DM and obesity on HDL efflux capacity, particle size and HDL sub-fraction proteomic composition, to gain greater understanding of the residual CVD risk present in these high-risk populations that is not attributable to traditional risk factors. We found that different proteins are associated with HDL-CEC conditional on T1DM or T2DM status e.g., HDL-associated ApoA-I and ApoA-II lost their association with HDL-CEC in the setting of diabetes. We also demonstrated that separation of HDL into large and small particles provided greater insight into the association of HDL proteins with ABCA1-independent and ABCA1-dependent CEC.

Lastly, in Chapter 5, we examined sex-specific differences in HDL protein composition and function in people with and without T1DM, T2DM and obesity to gain greater insight into potential mechanisms driving loss of the female 'advantage' against CVD in the setting of diabetes. We report that women with both T1DM and T2DM are disadvantaged in terms of HDL-CEC relative to male counterparts. We also report sex-specific differences in the proteins governing HDL-CEC in women versus men that may have important implications for their respective CVD risk profile. Indeed, our findings indicate the use of separate biomarkers to predict HDL function in men and women.

Our findings have collectively demonstrated that the HDL proteome tightly aligns with HDL-CEC, but proteins governing HDL-CEC differ in those with and without diabetes, and also differs between men and women. We have further demonstrated that measuring the proteomic composition of large versus small HDL-P, and in turn the function of large and small HDL-P was critical to truly untangle the complex relationship between HDL structure and function.

Chapter 1
Introduction

Abbreviations

7-KC	7-Ketocholesterol
ABCA1	ATP-Binding Cassette Subfamily A Member 1
ABCG1	ATP-binding cassette subfamily G, member 1
ACCELERATE	Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition with Evacetrapib in Patients with a High Risk for Vascular Outcomes
ACN	Acetonitrile
AGE	Advanced Glycation End Products
Apo	Apolipoprotein
ApoA-I	Apolipoprotein A-I
BP	Blood Pressure
BSA	Bovine Serum Albumin
CACTI	Coronary Artery Calcification in Type 1 Diabetes
CAD	Coronary Artery Disease
cAMP	Cyclic Adenosine Monophosphate
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcome Study
CE	cholesterol esters
CEC	Cholesterol Efflux Capacity
CETP	Cholesterol Ester Transfer Protein
CI	Confidence Interval
CKD	Chronic Kidney Disease
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
CYP7A1	Cholesterol 7α-Hydroxylase
dal-OUTCOMES	Effects of the Cholesterol Ester Transfer Protein Inhibitor Dalcetrapib in Patients with Recent Acute Coronary Syndrome
DGUC	Density Gradient Ultracentrifugation
DKA	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DTT	Dithiothreitol
EDIC	Epidemiology of Diabetes Interventions and Complications
EDTA	Ethylenediaminetetraacetic Acid
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Regulated Kinase
ER- α	Oestrogen Receptor α

ERN	Extended-Release Niacin
FA	Formic Acid
FAA	Free-Fatty Acids
FC	Free Cholesterol
FH	Familial Hypercholesterolaemia
FPLC	Fast Protein Liquid Chromatography
GADA	65kDa Glutamic Acid Decarboxylase Autoantibodies
GAPDH	Glyceraldehyde-3-Dehydrogenase
GLP-1	Glucagon-like Peptide 1
GLUT-4	Glucose Transporter Type 4
GWAS	Genome Wide Association Studies
HAEC	Human Aortic Endothelial Cells
HbA1c	Haemoglobin A1c
HDL	High-Density Lipoprotein
HDL-P	High-Density Lipoprotein Particles
HR	Hazard Ratio
HUVEC	Human Umbilical Vein Endothelial Cells
IAA	Iodoacetamide
ICAM-1	Intercellular Adhesion Molecule 1
IDL	Intermediate-Density Lipoprotein
IL	Interleukin
ILLUMINATE	Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
JUPITER	Justification for the Use of Statins in Prevention: An Intervention Trail Evaluating Rosuvastatin
LADA	Latent Autoimmune Diabetes of the Adult
LCAT	Lecithin-Cholesterol Acyltransferase/ Phosphatidylcholine-sterol Acyltransferase
LDL	Low-Density Lipoprotein
LDLR	LDL-Receptor
LFQ	Label-Free Quantification
LOOH	LDL-Associated Lipid Hydroperoxides
LOH	Lipid Hydroxides
LPC	Lysophosphatidylcholine
LPL	Lipoprotein Lipase

LPS	Lipopolysaccharide
LRP	LDL Receptor-Related Protein
LRA	Lipid Removal Agent
MARD	Mild Age-Related Diabetes
MCP-1	Monocyte Chemoattractant Protein-1
MEM	Minimum Essential Media
MetS	Metabolic Syndrome
MHO	Metabolically Healthy Obese
MI	Myocardial Infarction
MOD	Mild Obesity-Related Diabetes
MODY	Maturity Onset Diabetes of the Young
MS	Mass Spectrometry
MUO	Metabolically Unhealthy Obese
NCEP-ATP III	National Cholesterol Education Program – Adult Treatment Panel III
NCP1L1	Niemann-Pick Type C1 -like 1
NF-κB	Nuclear Factor-Kappa B
NO	Nitric Oxide
OR	Odds Ratio
oxLDL	Oxidised LDL
PAD	Peripheral artery disease
PARP	Poly(ADP-Ribose) Polymerase
PAF-AH	Platelet Activating Factor Acethyldrolase
PBS	Phosphate-Buffered Saline
PC	Phosphatidylcholine
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PE	Phosphatidylethanolamine
PEG	Polyethylene Glycol
PGI_2	Prostacyclin
PKC	Protein Kinase C
PL	Phospholipids
PLTP	Phospholipid Transfer Protein
PON1	Paraoxonase 1/ Serum Paraoxonase/Arylesterase 1
RCT	Reverse Cholesterol Transport
REVEAL	Randomized Evaluation of the Effects of Anacetrapib through Lipid modification
rHDL	Reconstituted HDL
ROS	Reactive Oxygen Species

RR	Relative Risk
S1P	Sphingosine 1-Phosphate
SAA	Serum Amyloid A
SAID	Severe Autoimmune Diabetes
SD	Standard Deviation
sdLDL	Small, Dense LDL
SIDD	Severe Insulin-Deficient Diabetes
SIRD	Severe Insulin-Resistant Diabetes
SM	Sphingomyelin
SMC	Smooth Muscle Cells
SR-B1	Scavenger Receptor Class B Type 1
SVUH	St. Vincent's University Hospital
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TRL	Triglyceride Rich Lipoproteins
TLR-4	Toll-like Receptor 4
TUH	Tallaght University Hospital
VADT	Veteran Affairs Diabetes Trial
VCAM-1	Vascular Cell Adhesion Molecule-1
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organisation

1.1 Diabetes mellitus (DM)

The International Diabetes Federation estimate that one in ten adults have diabetes mellitus (DM) globally, equivalent to 537 million adults between 20 and 79 years of age. By 2045, there will be an estimated 783 million adults with DM. DM has devasting effects on individuals and societies. It resulted in approximately 6.7 million deaths worldwide in 2021, which corresponds to 12.2% of global deaths from all causes in the 20-79 year age group [1].

The estimated prevalence of DM in women aged 20-79 years is slightly lower than in men (10.2% vs. 10.8%) [2]. Both clinical and experimental studies indicate that post-pubertal sex steroid hormones largely contribute to the sex differences observed in DM susceptibility. The protective role of endogenous oestrogen in women is evidenced by the deleterious impact of the menopause on body composition and glucose homeostasis, leading to an increased incidence of metabolic disorders compared to premenopausal women [3]. Additionally, rare loss-of-function mutations in the gene encoding either aromatase, the enzyme that converts androgens to oestrogens, or oestrogen receptor α (ER-α) leads to dysmetabolic phenotypes in individuals of both sexes [4], further highlighting the contribution of the oestrogen pathway to DM susceptibility.

DM is broadly categorised into type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM), which are complex heterogeneous diseases. Other types of DM exist which are expanded on in **Section 1.1.1**. T1DM is characterised by autoimmune pancreatic β-cell destruction, normally resulting in absolute insulin deficiency [5]. T2DM is defined as the progressive loss of pancreatic β-cell function to secrete insulin combined with insulin resistance (IR) in target organs [5, 6]. T2DM is the most common form, affecting 90% of people with DM [7]. Insulin, a pleiotropic hormone, is a central regulator of blood glucose with normoglycaemia being maintained through tightly controlled insulin action and secretion. Pancreatic β-cells secrete insulin, which drives anabolic metabolism while decreasing catabolic metabolism [8].

The primary, clinical, and diagnostic characteristic of DM is hyperglycaemia [5]. The American Diabetes Association define the criteria for diagnosing DM based on plasma glucose, either fasting plasma glucose, or 2 hour plasma glucose during a 75g oral glucose tolerance test, or glycosylated haemoglobin (HbA1c) test [5]. **Table 1.1** depicts the diagnosis criteria for DM and prediabetes.

Table 1.1 *Diagnosis criteria for diabetes and prediabetes [5].*

	Prediabetes	**Diabetes**
Fasting plasma glucose	100 – 125 mg/dL (5.6 – 6.9 mmol/L)	≥126 mg/dL (7.0 mmol/L) Fasting defined as no caloric intake for at least 8 hours
2-hour plasma glucose	140 – 199 mg/dL (7.8 mmol/L – 11.0 mmol/L)	≥200 mg/dL (11.1 mmol/L) during OGTT
HbA1c test	5.7 – 6.4% (39-47 mmol/mol)	≥6.5% (48 mmol/mol)
Other		In people with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).

**Testing needs to be completed on two samples, and two abnormal results obtained for diabetes to be diagnosed.

1.1.1 Diabetes disease heterogeneity

Distinguishing people with T1DM from those with T2DM is not clinically clear-cut. There are subtypes of DM that don't fit neatly into these two main categories and are highlighted in **Table *1.2***. No standard case definitions exist for research or surveillance of DM. Terms such as 'type 1.5 diabetes', 'double', 'hybrid' or 'mixed' diabetes have been and continue to be used [9].

Table 1.2 *Diabetes subtypes.*

Diabetes subtype	**Characteristics**
Latent autoimmune diabetes of the adult (LADA)	• Defined by the presence of 65kDa glutamic acid decarboxylase autoantibodies (GADA). • Anti-islet autoimmunity, and IR suggesting the 'co-existence' of T1DM and T2DM [10].
Maturity onset diabetes of the young (MODY)	• Monogenic form of diabetes [11]. • Autosomal dominant inheritance, young age of onset (normally 20-40 years of age), and continued secretion of endogenous insulin [11]. • Similar to T1DM in that both present in lean individuals at a young age [12, 13]. • Similar to T2DM in that both retain endogenous insulin secretion, and it's not usually associated with β-cell antibodies [12, 13].

In 2018, a redefined classification system aimed to identify individuals with an increased risk of complications associated with DM, and to better understand the underlying mechanisms of the disease [14]. The clusters, shown in **Table *1.3***, were based on six variables: GADA, age at diagnosis, body mass index (BMI), HbA1c, and homeostatic model assessment (HOMA) 2 estimates of β-cell function and IR. This new sub stratification might eventually help to customise and target early treatment to people who would benefit most, thereby enabling the first steps towards precision medicine in DM [14].

Table 1.3 *Novel subtypes of DM classified into clusters [14].*

Diabetes subtype	Characteristics
Severe autoimmune diabetes (SAID)	• Traditionally classed as T1DM. • Typically have early-onset disease, low BMI, poor metabolic control, GADA, and insulin deficiency.
Severe insulin-deficient diabetes (SIDD)	• Similar to SAID but GADA negative.
Severe insulin-resistant diabetes (SIRD)	• Elevated IR and high BMI.
Mild obesity-related diabetes (MOD)	• High BMI, but lower IR and younger age relative to the SIRD group.
Mild age-related diabetes (MARD)	• Older in age, with lower BMI and less IR, relative to SIRD/MOD groups • Present with milder metabolic syndrome (MetS) compared to other groups.

For the purposes of this thesis the 'traditional' categorisation of T1DM and T2DM will be used as that is what is still utilised in the clinical setting and continues to be widespread in the scientific literature.

1.1.2 Type 1 diabetes mellitus (T1DM)

T1DM is the most common form of DM in children and adolescents, and over 1.2 million currently live with T1DM worldwide [1]. T1DM is an autoimmune disease. The pathogenesis for T1DM put forward is a continuum that can be separated into stages related to the detection of autoantibodies, dysglycaemia, and lastly, symptoms linked to hyperglycaemia [9], such as polydipsia, polyphagia, and polyuria [15]. See **Table 1.4**. Factors such as rapid longitudinal growth, puberty, low physical activity, being overweight, trauma, and infections, have been

suggested to enhance β-cell stress and thereby trigger progression to clinical onset of DM in children with β-cell autoimmunity [16].

The loss of the pancreatic β-cells is the outcome of T1DM-related autoimmunity, concurrent with the formation of T1DM-associated autoantibodies many months or years before symptom onset [9]. The antigen presentation of B cells and dendritic cells promotes the activation of β-cell-specific T cells. The exposure of B cells to β-cell autoantigens leads to the generation of islet-targeting autoantibodies, which operate as biomarkers of asymptomatic disease [9] (**Figure 1.1**).

The primary islet cell autoantibodies include insulin autoantibodies (IAA), 65kDa glutamic acid decarboxylase autoantibodies (GADA), insulinoma antigen-2 autoantibodies (IA-2A), islet cell autoantibody 512 (ICA512) and zinc transporter 8 autoantibodies (ZnT8A) [17]. The presence of multiple autoantibodies greatly enhances the likelihood for developing T1DM – over 70% of T1DM have three or four autoantibodies, while less than 5% have only a single autoantibody [18].

Dysglycaemia indicates the progression from stage 1 to stage 2. When enough functional β-cell mass has been destroyed, biochemical tests can reveal impaired glucose tolerance even though no symptoms are present [16]. The pathogenic effects of hyperglycaemia results in tissue damage through 5 main mechanisms: (1) Increased flux of glucose and other sugars through the polyol pathway; (2) Increased intracellular formation of advanced glycation end products (AGE); (3) Increased expression of the receptor for AGE and its activating ligands; (4) Activation of protein kinase C isoforms; and (5) Overactivity of the hexosamine pathway [19] (**Figure 1.2**).

All 5 mechanisms are activated by the mitochondrial electron transport chain overproducing superoxide, resulting in oxidative stress, and increasing the production of reactive oxygen species (ROS) [20]. This has multiple downstream effects, such as causing DNA strand breakages [19]. DNA damage activates poly(ADP-ribose) polymerase (PARP), which then makes polymers of ADP-ribose. These polymers alter the activity of glyceraldehyde-3-dehydrogenase (GAPDH), which impairs glycolysis, and diverts glycolytic intermediates into pathogenetic signalling pathways [9, 19]. Hyperglycaemia can also promote inflammation through AGE-modified proteins binding to their surface receptors, increasing the release of proinflammatory cytokines, and stimulating inflammatory pathways in vascular endothelial cells [21]. The presence of overt symptoms is indicative of T1DM.

Although insulin treatment improves glucose metabolism, the DM-related disease burden increases with time for the development of chronic complications (See **Sections 1.2**).

Table 1.4 *Staging of T1DM [9].*

	Stage 1	Stage 2	Stage 3
β-cell autoimmunity (with the presence of autoantibodies)	Present	Present	Present
β-cell loss	Present	Present	Present
Dysglycaemia	Absent	Hyperglycaemia	Hyperglycaemia
Symptoms	Absent	Absent	Present

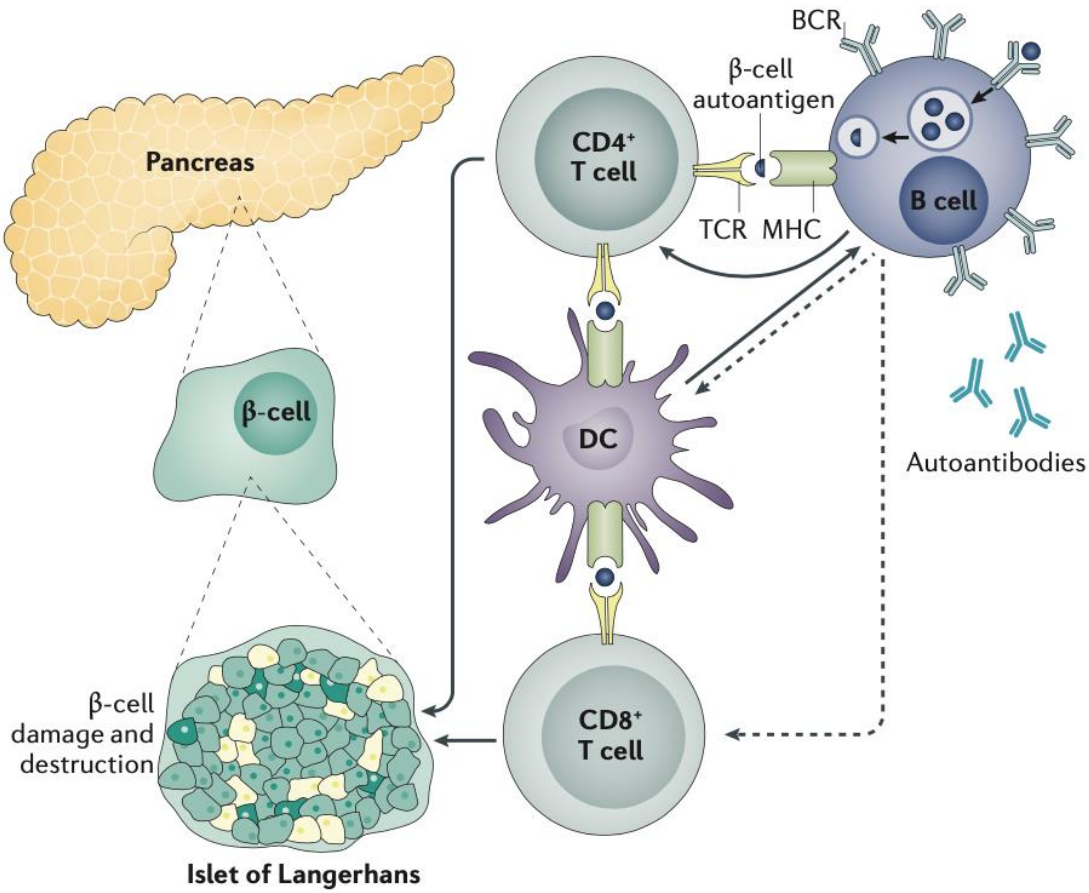


Figure 1.1 *Pathogenesis of T1DM.*
Activated B cells interact with $CD4^+$ and $CD8^+$ T cells, as well as dendritic cells (DC). B cells and DC antigen presentation promotes the activation of β-cell-specific T cells. The exposure of B cells to β-cell autoantigens results in the generation of islet-targeting autoantibodies, which are used as biomarkers of asymptomatic disease. Dashed arrows indicate the possible interactions between B cells and $CD8^+$ T cells, and between B cells and DCs.
Image taken from Katsarou, A., *et al.,* [9].

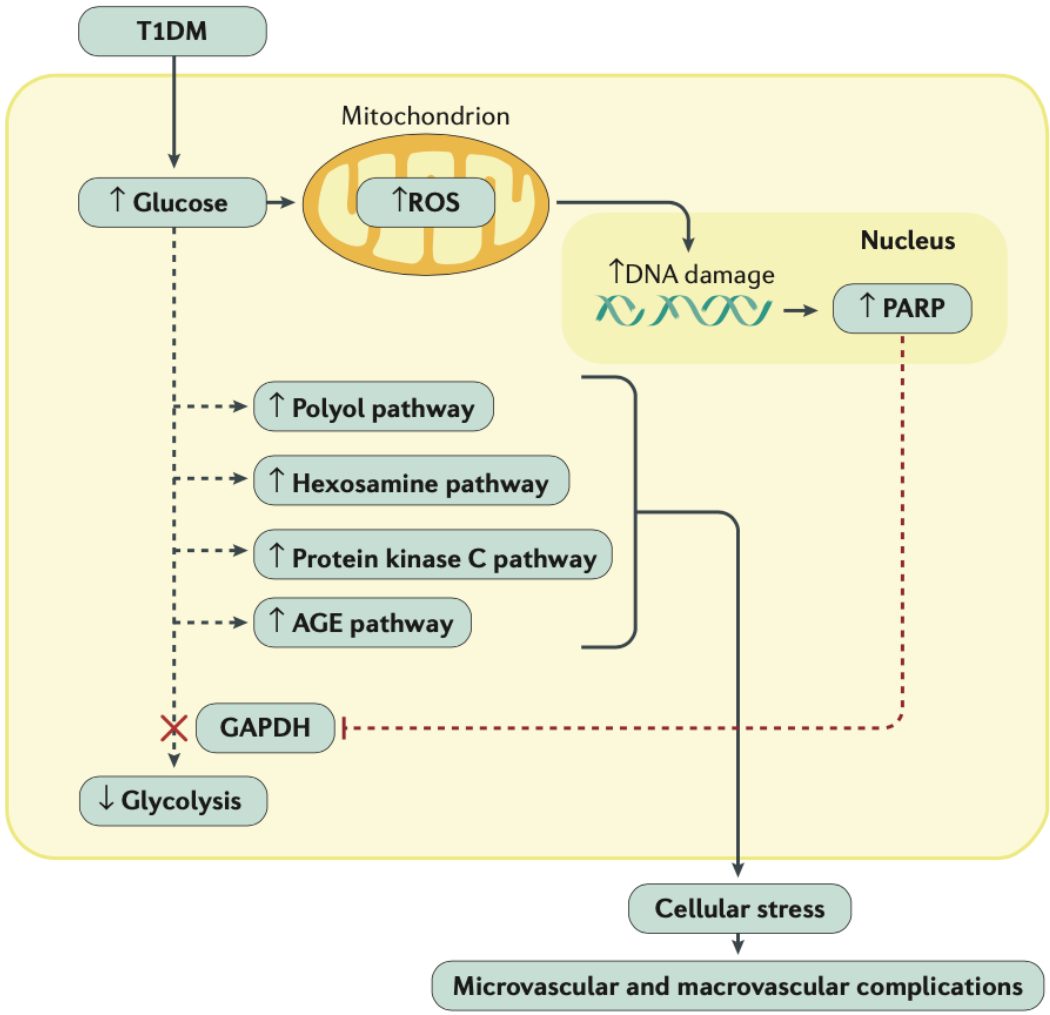


Figure 1.2 *Mechanisms of hyperglycaemia-induced cellular damage.*
Elevated intracellular glucose levels results in oxidative stress, and the increased production of ROS, which have effects, such as causing DNA strand breaks. DNA damage activates PARP, which then makes polymers of ADP-ribose. These polymers bind to and modify the activity of GAPDH. Blocking GAPDH leads to a bottleneck in glycolysis, such that glycolytic intermediates are diverted into pathogenetic signalling pathways (dashed arrows). Image taken from Katsarou, A., *et al.,* [9].

1.1.3 Type 2 diabetes mellitus (T2DM)

The worldwide rise in obesity, sedentary lifestyles, and an ageing population, has led to the near quadrupling of the incidence and prevalence of T2DM globally [1, 22]. T2DM is a multifactorial disease involving both biological, and environmental factors. Risk factors for T2DM include family history of DM, unhealthy diet, physical inactivity/ sedentary behaviour, increasing age, hypertension, ethnicity, impaired glucose tolerance, history of gestational DM, poor nutrition during pregnancy, and smoking. However, the most important risk factor for T2DM is increasing adiposity [6, 7].

The central feature of T2DM is the loss of control of blood glucose levels resulting in hyperglycaemia. IR in muscle, adipose and liver, combined with the impairment of pancreatic β-cells to secrete insulin explain the core pathophysiological changes. The ‘triumvirate’ idea that T2DM results from defects in muscle, liver and pancreatic β-cells was proposed by

DeFronzo in 1987 [23]. By 2009, DeFronzo expanded the model to include eight definite pathophysiological abnormalities, dubbed the 'ominous octet', that participate in impaired glucose homeostasis, all of which are present early in the natural history of T2DM [24]. The model includes adipose tissue, gastrointestinal tract, pancreatic α cells, kidneys, and brain, in addition to the muscle, liver and β-cells [24].

IR in adipocytes leads to enhanced lipolysis, and therefore increased levels of plasma free-fatty acids (FFA), both of which exacerbate IR in muscle and liver, and contribute to β-cell failure [24]**.** In the gut, the incretin hormone glucagon-like peptide 1 (GLP-1), which functions to stimulate insulin secretion and inhibit glucagon secretion, is reduced in T2DM [25]. β-cell resistance to GLP-1 participates in the progressive failure of β-cell function, with consequent elevated glucagon (produced by pancreatic α cells) levels combined with greater hepatic sensitivity to glucagon, contributing to the excessive glucose production in the liver [26]. Increased renal glucose reabsorption along with the raised threshold for glucose spillage in the urine support the sustenance of hyperglycaemia [27]. Resistance to appetite-suppressive effects (GLP-1 effects) combined with low brain dopamine levels, and increased brain serotonin levels aid weight gain, which aggravates the underlying IR. In addition, vascular IR and activation of inflammatory pathways have been added to the 'ominous octet', creating the 'decadent decoplet' [6] (**Figure 1.3**).

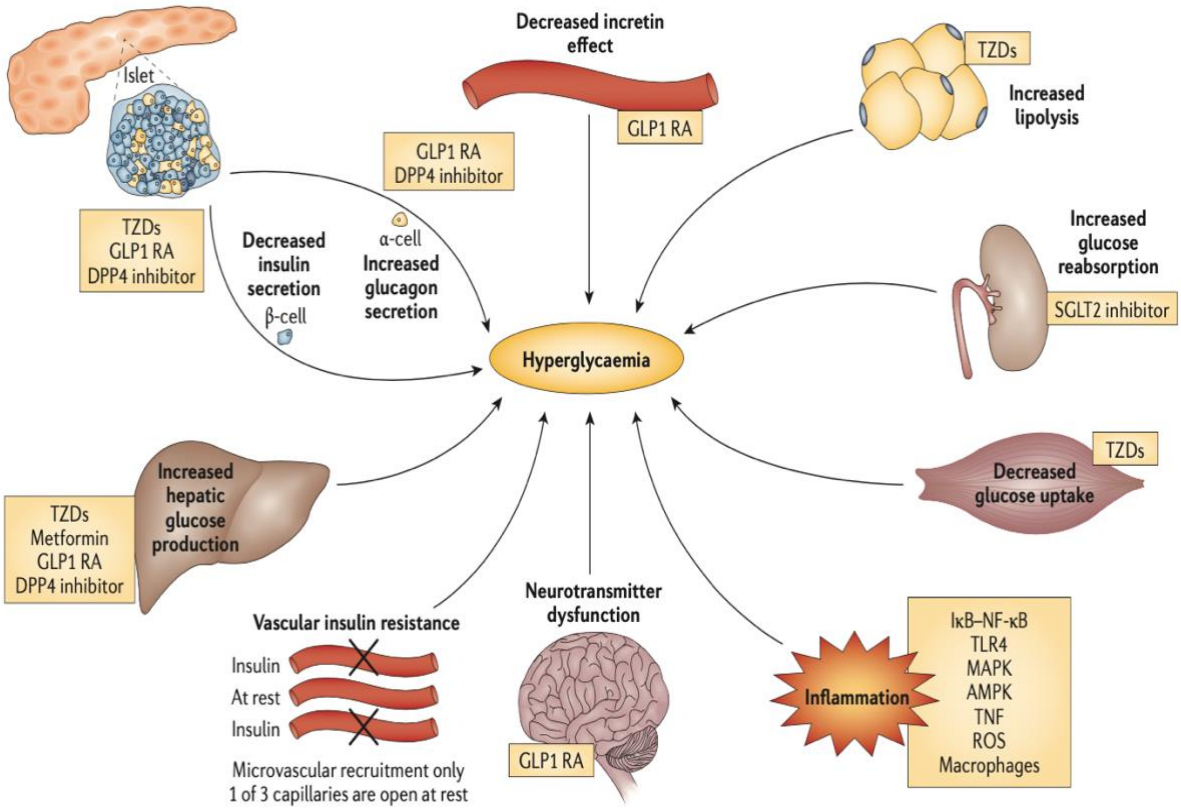


Figure 1.3 *The 'decadent decoplet' of hyperglycaemia in T2DM [6].*
The core defects in T2DM are IR in muscle and the liver, and impaired insulin secretion by the pancreatic β-cells. β-cell resistance to GLP1 contributes to progressive failure in the function of β-cells, whereas elevated glucagon levels and enhanced hepatic sensitivity to glucagon contribute to the excessive glucose production by the liver. IR in adipocytes results in accelerated lipolysis and elevated plasma FFA levels, both of which aggravate the IR in muscle and the liver and contribute to β-cell failure. Increased renal glucose reabsorption by the sodium/glucose co-transporter 2 (SGLT2) and the increased threshold for glucose spillage in the urine contribute to the maintenance of hyperglycaemia. Resistance to the appetite-suppressive effects of insulin, leptin, GLP1, amylin and peptide YY, as well as low brain dopamine and increased brain serotonin levels contribute to weight gain, which exacerbates the underlying resistance. Vascular IR and inflammation, must be added to the 'ominous octet' making the 'decadent decoplet'.
Image taken from DeFronzo, R.A., *et al.,* [6].

1.1.4 Obesity

Overweight and obesity are present day epidemics affecting 1.9 billion people worldwide [28]. Obesity is defined as a disproportionate body weight for height with excessive accumulation of adipose tissue that is frequently accompanied by mild, chronic systemic inflammation (metaflammation) [29]. The World Health Organisation (WHO) classifies an individual as overweight if they have a BMI between 25-29.99 kg/m^2, and obese if their BMI is >30 kg/m^2. Obesity is associated with multiple co-morbidities including T2DM, cardiovascular disease (CVD) and certain types of cancer [28]. These health complications can contribute to the premature death of individuals with obesity compared to individuals who are normal weight [30]. Obesity and T2DM are inter-related risk factors for each other and for the development of CVD. The association between obesity and incidence of T2DM in men is a rate ratio (RR) of 6.74 (95% CI 5.55, 8.19), while in women it is 12.41 (95% CI 9.03, 17.06) [31]. The

association between obesity and coronary artery disease (CAD) in men is a RR of 1.72 (95% CI 1.51, 1.96), while in women it is 3.1 (95% CI 2.81, 3.43) [31]. These studies highlight that incidence of associated complications of obesity are higher in women compared to men, however the mechanisms underpinned these are not fully elucidated.

Notably, not all individuals with obesity develop health complications, however, assessing those at high and low risk is a challenge, and tests to achieve this are non-existent [32, 33]. The MetS is a cluster of metabolic abnormalities that include central obesity, IR, hypertension, and an atherogenic lipid profile where fasting triglycerides (TG) levels are increased, and high-density lipoprotein-cholesterol (HDL-C) levels are decreased. The National Cholesterol Education Program – Adult Treatment Panel III (NCEP-ATP III) established criteria to classify MetS (**Table 1.5**) [34]. People with obesity having three or more parameters are classed as metabolically unhealthy obese (MUO), while those having less than three are classified as metabolically healthy obese (MHO). However, the prognostic value of MHO is hotly debated, mainly because it likely shifts gradually towards MUO, indicating that it is probably a transient condition [35, 36]. There are no universally accepted criteria to identify MHO, hence its prevalence varies widely among studies. Furthermore, MHO individuals are still at higher risk of CVD than normal weight metabolically healthy individuals [37]. MHO is more often observed in young, physically active people with a better nutritional status and low levels of ectopic and visceral fat storage [38].

Therefore, it's difficult to identify individuals with obesity who are at highest risk of developing health complications. Better understanding of the mechanisms linking obesity to associated complications, and identifying sensitive biomarkers of disease risk will be critical for advising clinical decision making.

Table 1.5 *NCEP-ATP III criteria for Metabolic syndrome [34].*

Risk Factor	Guideline
Waist Circumference	Men > 102 cm Women > 88 cm
Fasting Blood Glucose	≥ 100 mg/dL
Blood Pressure	≥ 130/≥ 85 mmHg
Triglycerides	≥ 150 mg/dL
HDL-C	Men < 40 mg/dL Women < 50 mg/dL

1.1.5 Obesity and Insulin resistance (IR)

Adipose tissue has a central role in the adaption of energy metabolism to the nutritional environment because of its ability to store energy as TG. In obesity, the hormone insulin enables surplus lipid to be stored in adipocytes as TG to prevent a lipotoxic environment.

Insulin signalling drives translocation of glucose transporter type 4 (GLUT-4) to the adipocyte cell surface, which then facilitates glucose uptake into the cell. This simultaneously reduces the secretion of FFA by inhibiting lipolysis, and increases TG synthesis [39, 40]. Adipocyte tissue expansion can happen in one of two ways: through enlargement of existing adipocytes (hypertrophy) to accommodate TG storage or through the proliferation of pre-adipocytes and differentiation into mature adipocytes (hyperplasia) [41]. The excessive TG aggregation, and cholesterol imbalances, can trigger and sustain inflammation in adipocytes, disrupting metabolic homeostasis [42]. The recruitment of *in situ* inflammatory cells such as macrophages into adipose tissue further exasperates the inflammatory burden in the obese setting [43], as within obese adipose tissue these macrophages undergo a phenotypic switch from anti-inflammatory/ pro-resolving M2 macrophages towards pro-inflammatory M1 macrophages, which contributes to IR [44]. Hyperglycaemia and increased circulating FFA follow after progressive IR, promoting a chronic inflammatory state [45].

IR precedes the development of T2DM by many years [46]. Insulin stimulates tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, which is pivotal in mediating insulin action [47]. In IR, this step is usually defective, and causes are multifactorial including ectopic lipid accumulation, mitochondrial dysfunction, endoplasmic reticulum stress and inflammation [6]. For example, TNFα and IL-1β target this process through inhibiting serine phosphorylation of IRS-1 [47], while lack of IL-1 receptor protects against high-fat diet induced insulin resistance in mice [48]. Chronic exposure to high glucose levels, and FFA stimulates islet inflammation, and combined with macrophage recruitment, can lead to increased β-cell apoptosis, and impaired insulin secretion [49, 50]. This can drive the advancement from obesity, and IR to overt T2DM [49].

1.2 Diabetes complications

Thanks to the discovery of insulin treatment 100 years ago, the natural history of DM has changed. People with DM are not dying of hyperglycaemia per se. but suffer substantial morbidity and mortality owing to the chronic complications associated with their disease.

People with T2DM are at a two-fold increased risk of death [51]. The excess risk of death in T2DM compared to controls from any cause and cardiovascular death increases with younger age, poorer glycaemic control, and greater severity of renal complications [51]. People with T1DM are at a two to ten-fold increased risk of death, largely due to CVD, which depends on their HbA1c levels, with higher levels indicating higher risk [52]. This translates into a loss of life expectancy at 20 years of approximately 12 years relative to people without DM [52, 53]. Understanding and monitoring diabetic complications are necessary for their successful

treatment. The complications of DM are broadly split into microvascular and macrovascular complications and are depicted in **Figure 1.4**.

1.2.1 Microvascular complications

1.2.1.1 Diabetic retinopathy, Diabetic nephropathy and Diabetic neuropathy

Diabetic retinopathy has an approximate 30% prevalence rate in DM, with 5-10% experiencing sight-threatening stages, and diabetic macular oedema [54, 55]. Retinopathy is strongly linked to prolonged duration of DM, hyperglycaemia and hypertension [56].

Diabetic nephropathy is a primary cause of chronic kidney disease (CKD) [57]. The prevalence of renal dysfunction in T2DM is 42%, compared to 32% in T1DM [58]. Nephropathy is recognised when urinary albumin excretion is elevated in the absence of other renal conditions. The severity of nephropathy is characterised in relation to the degree of albuminuria. Microalbuminuria is defined as an albumin excretion rate of 30–299 mg / 24 hours. Microalbuminuria may progress to macroalbuminuria, which is defined as an albumin excretion rate of ≥300 mg / 24 hours [57]. Macroalbuminuria can be followed by a persistent decline in kidney function, renal impairment, and finally end-stage renal disease [57].

Diabetic neuropathy is a collection of syndromes involving the peripheral and autonomic nervous systems. Distal symmetric polyneuropathy occurs in approximately 20% of T1DM after 20 years disease duration, and in 10-15% of newly diagnosed T2DM, with rates elevating to 50% after 10 years of disease [59]. There is a loss of sensory function starting distally in the lower extremities that is also characterized by pain and substantial morbidity [60].

1.2.2 Macrovascular complications

1.2.2.1 Peripheral artery disease (PAD)

Peripheral artery disease (PAD) is frequently a manifestation of atherosclerosis, where an atherosclerotic plaque causes stenosis or occlusion. This leads to reduced blood flow in the affected limb. Most people are asymptotic but often encounter intermittent claudication (pain on walking) [61]. The risk of intermittent claudication is two to three-fold higher in people with DM compared to those without [62]. The risks of PAD increase with the severity of DM: for every 1% increase in HbA1c, the risk of PAD goes up by 32% in T1DM, and 28% in T2DM [63]. The risk of major amputation is around five-fold higher in people with DM [64].

1.2.2.2 Cerebrovascular disease

Stroke is defined as an acute episode of focal dysfunction caused by vascular pathology in the brain or in the vessels leading to and from the brain such as the retina or spinal cord, which lasts longer than 24 hours, or of any duration if imaging or autopsy display focal infarction or

haemorrhage [65]. Stroke is divided into two main subtypes: ischemic and haemorrhagic. Stoke occurs twice as often in people with DM compared to those without [66].

1.2.2.3 Cardiovascular disease (CVD)

CVD and DM are in the top 10 causes of death globally [67], comprising an immediate risk to public health and welfare worldwide. CVD is the leading cause of mortality globally, with the WHO estimating that 17.9 million people died from CVD in 2019, representing 32% of worldwide deaths [68]. The Pittsburgh Epidemiology of Diabetes Complications study of T1DM reported cardiovascular events in adults younger than 40 years of age to be 1% per year, and three times higher in individuals over 55 years [69]. Moreover, a meta-analysis concluded that CVD affects approximately 32.2% of all people living with T2DM, with the primary mortality causing sub-types of CVD being CAD and stroke [70]. However, a major confounder in people with T2DM is the presence of the MetS. A high percentage of these people are obese, and prevalence of hypertension is also higher. Notably, the duration of exposure to high glucose levels is much longer in T1DM compared to T2DM. Thus, while both T1DM and T2DM are associated with increased risk for CVD, it is highly likely the underlying mechanisms driving CVD are different for both diseases.

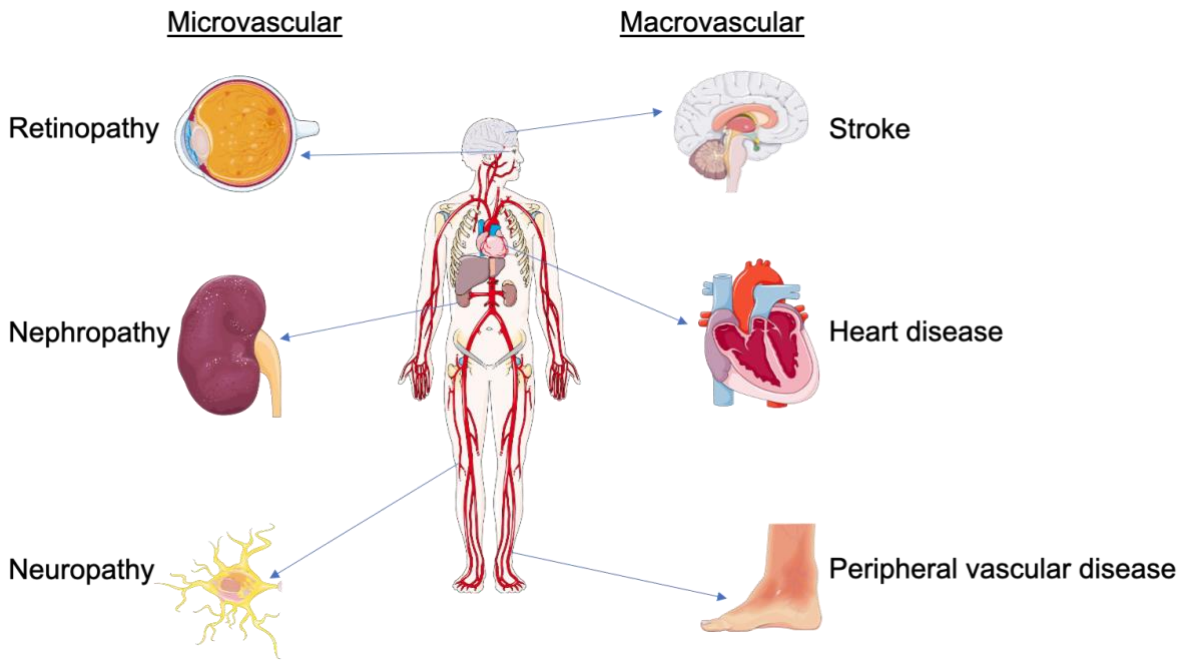


Figure 1.4 *Major Complications of Diabetes.*
Microvascular and macrovascular complications of diabetes.
Image prepared using SMART – Servier Medical ART and Microsoft PowerPoint.

1.3 Sex differences in risk of CVD in diabetes

Another important point to note in DM is a particularly strong preponderance to increased CVD risk in women relative to men with the disease. Women without DM are relatively protected

from CVD compared to men without DM, with lower mortality rates from coronary heart disease (CHD) evident [71]. Oestrogen and testosterone can play a differential role in atherosclerotic risk factors as depicted in **Figure 1.5**. The vascular actions of oestrogen are predominately mediated via ER-α signalling promoting an anti-inflammatory, low-vascular resistance phenotype that is protected from CVD [72]. However, the cardioprotective benefits associated with being a woman are negated in women with DM through as yet unknown mechanisms. Women with DM are more severely impacted by their disease compared to men [73]. A meta-analysis of 47 cohorts including 12 million individuals reported a multiple adjusted relative risk (RR) for heart failure associated with T2DM as 1.95 (95% CI, 1.70, 2.22) in women and 1.74 (95% CI, 1.55, 1.95) in men [73]. Similarly, the corresponding RR associated with T1DM were 5.15 (95% CI 3.43, 7.74) for women and 3.47 (95% CI 2.57, 4.69) for men [73]. As a result of the increased risk of CVD in women with DM, this has translated into more deaths associated with DM in women (2.3 million) than in men (1.9 million) worldwide [2]. Possible explanations for this imbalance of risk include undertreatment of women with DM [74], and that historically, women with DM had poorer glycaemic control than men with DM [75].

However, despite growing differences demonstrating sex and gender differences in baseline CVD risk factors, coronary anatomy and function, symptoms presentation, comorbidities, treatment efficacy and outcomes of CVD, the mechanisms behind these differences are largely unexplored [76, 77]. These knowledge gaps are sustained by the persistent underrepresentation of women in CVD trials and a lack of basic science evidence obtained from female animals and cells due to the manifold refuted claim that inclusion of females will increase variability, as well as double sample size and costs [78-82]. Therefore, a key focus of this thesis was to understand the mechanisms driving increased risk of CVD not only in men and women with DM combined relative to the general population, but also in women with DM relative to men with DM.

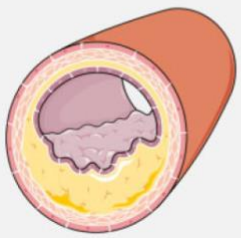
	Estrogens	Testosterone
Atherosclerosis Risk Factors 	LDL oxidation↓ LDL binding↓ VSMC proliferation↓ VSMC migration↓ EC proliferation↑ EC migration↑ CRP↑ Pro-inflammatory (TNF-α, IFNγ, IL-6, CCL2) cytokine production↑ Hematopoietic stem cell differentiation↑ Coronary calcification↓	Conflicting effects on lipids Expression of pro-atherogenic genes↑ WBC adherence to EC↑ Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ)↓ CRP↓ IL-10↑ Plaque volume↑, VCAM-1 expression↑ (controversial data) Coronary calcification↑

Figure 1.5 *Effects of oestrogen and testosterone on atherosclerosis risk factors [83].*
Effects of oestrogen and testosterone on plaque development and atherothrombosis.
Image taken from Haider *et al.,* [83].

1.4 Overview of the pathophysiology of CVD

Atherosclerosis, the most common underlying cause of CVD, is a chronic progressive CVD encompassing a cholesterol disorder, and an inflammatory disease that can manifest as myocardial infarction (MI), stroke, and cardiovascular death [84]. CVD is associated with increased markers of classical systemic inflammation such as C-reactive protein (CRP), TNF-α, interleukin (IL) -6, IL-18, serum amyloid A (SAA), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) [85-87].

Low-density lipoprotein (LDL) particles participate causally in atherosclerosis, however, how LDL causes atherosclerosis is not fully understood. In animal research models, oxidised low-density lipoprotein (ox-LDL) particles are pro-inflammatory, immunogenic and contribute to the initial stages of atherosclerosis [88]. However, little evidence supports the causal role of ox-LDL in humans [89]. Recent work supports the participation of cavelolin-1-dependent LDL transcytosis through the endothelium in experimental atherosclerosis [90]. Other potentially mechanisms through which LDL promotes atherosclerosis include LDL that aggregates in the intima in association with proteoglycan, or adaptive immune responses to native LDL [89].

In the initial stages of atherosclerosis, endothelial cells undergo inflammatory activation [91], enabling them to express a leukocyte adhesion molecule (e.g. VCAM-1) that interacts with cognate ligands. This promotes the rolling and eventually adherence/ infiltration of blood monocytes and lymphocytes to the endothelial layer [89]. Chemokines and chemoattractant proteins facilitate the recruitment of these inflammatory cells into the intima. Monocytes develop into tissue macrophages, and phagocytose lipids, thereby generating foam cells within the intima of the arteries [88] (**Figure 1.6**). These lipid-laden foam cells release inflammatory cytokines, ROS, and other mediators, and also contribute to lesion bulk [92]. As the disease advances, smooth muscle cells (SMC) proliferate and migrate towards the lesions, forming a fibrous cap, within which is the necrotic, lipid-rich remnants of the foam cells [93] (**Figure 1.7**). As the lesion grows, it can rupture, and form a thrombus, which commonly results in MI, stroke, and cardiovascular death [92].

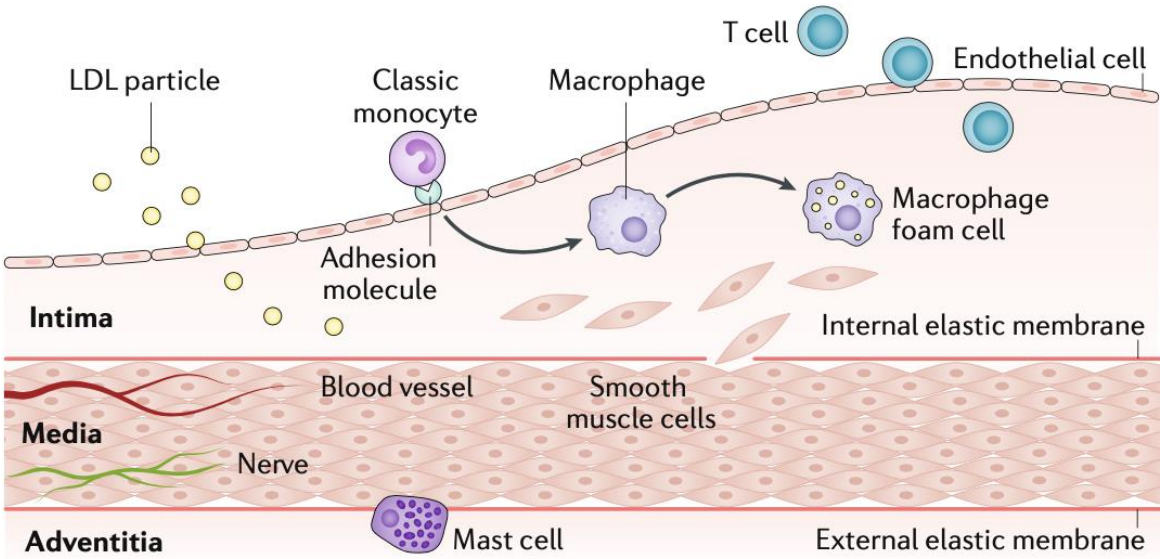


Figure 1.6 *Initiation and progression of atherosclerosis [88].*
The normal artery comprises three layers: the innermost intima (in close contact with the bloodstream), the tunica media, and the outer coat and adventitia. The atherosclerotic plaque forms in the innermost layer, the intima. In the early stage of lesion initiation, low-density lipoprotein (LDL) particles accumulate in the intima. Proinflammatory cytokines activate endothelial cells. The cells can then express leukocyte adhesion molecules that interact with cognate ligands to promote the rolling, and eventually adherence, of blood monocytes and lymphocytes to the endothelial layer. Chemoattractant cytokines can direct the migration of these bound leukocytes into the intima. Within the intima, foam cells form by uptake of lipids. Some of these lipid-laden foam cells arise from blood monocytes that have matured into macrophages. SMC (that are usually located in the media) can penetrate the intima. Image taken from Libby *et al.,* [88].

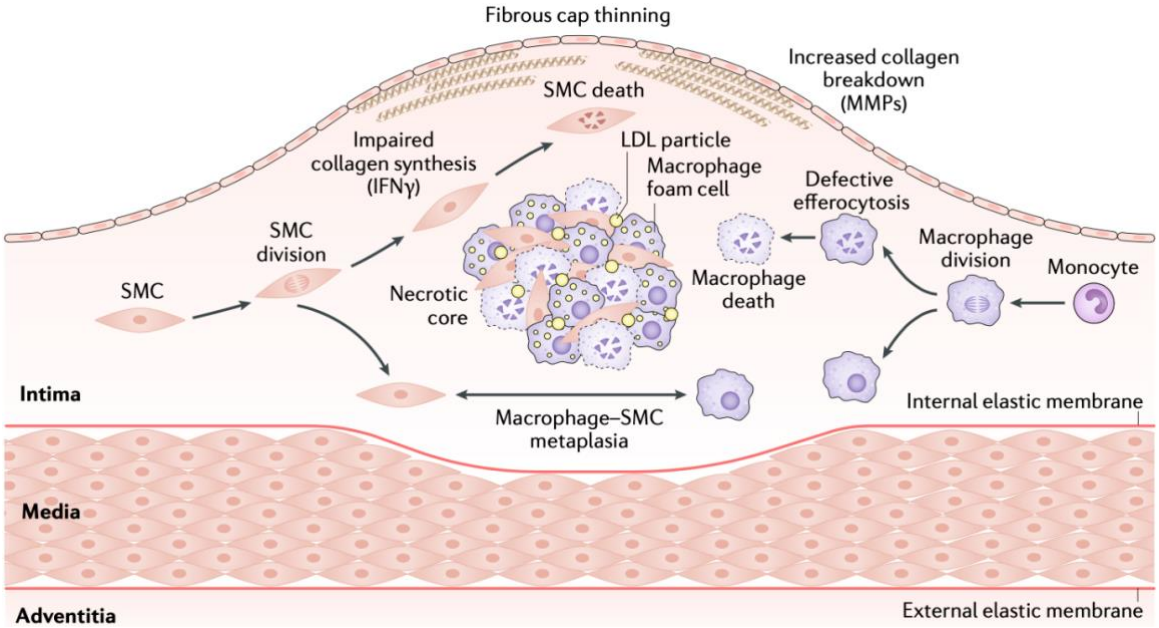


Figure 1.7 *Progression of atherosclerotic lesions [88].*
During the evolution of the atherosclerotic plaque, SMC produce extracellular matrix molecules (such as interstitial collagen and elastin) that contribute to the thickening of the intimal layer. However, T cell mediators such as IFNγ can impair the ability of the SMC to synthesize interstitial collagen and thereby dampen the ability of these cells to repair and maintain the fibrous cap that overlies the necrotic core. Furthermore, activated macrophages show increased production of enzymes of the matrix metalloproteinases family that degrade the interstitial collagen that lends strength to the fibrous cap. Thinning and structural weakening of the fibrous cap increase the susceptibility of the plaque to rupture. SMC and macrophages in the evolving lesion can divide. SMC and the mononuclear phagocytes can also interchange through a process of metaplasia. As the lesion advances, SMC and macrophages can undergo cell death including by apoptosis. The debris from dead and dying cells accumulates, forming the necrotic, lipid-rich core of the atheroma. Impaired efferocytosis (clearance of dead cells) can contribute to the formation of the necrotic core. Image taken from Libby *et al.,* [88].

1.4.1 Pathophysiology of CVD in diabetes

In DM, hyperglycaemia, excess FFA and IR promote oxidative stress, glyco-oxidation, and systemic inflammation, which work together to damage endothelial cells lining the arterial wall, and drive inflammation in the intimal layer of the coronary artery [94], thus initiating the atherosclerosis process (**Figure 1.8**). Endothelial dysfunction is a primary component of early atherosclerosis, whereby endothelium-dependent, nitric oxide (NO)-mediated, vasodilation is impaired [95, 96]. In general, NO potently dilates vessels, and mediates much of the endothelium control of vascular relaxation [97]. NO also inhibits platelet activation, limits inflammation by decreasing leukocyte adhesion to the endothelium, and reduces vascular SMC proliferation and migration [97-99]. These processes, together, inhibit atherosclerosis and protect the blood vessel. Insulin can directly stimulate NO release from the endothelium, however, this is impaired in DM due to lack of insulin/ endothelium IR [100]. Additionally, hyperglycaemia blocks endothelial nitric oxide synthase (eNOS) activation, thereby inhibiting NO production, and elevating the production of ROS in the endothelium, and vascular SMC [101]. The liberation of excess FFA from the adipose tissue due to IR activates the signalling enzyme protein kinase C (PKC), inhibiting an eNOS agonist pathway, and elevating the production of ROS [102], all of which directly impair NO production or reduce its bioavailability. These small microinsults, recurring over time, together with abnormal platelet activity, and fibrin deposition, predisposes people with DM to progressive luminal narrowing, producing impaired blood flow, and chronic ischemia, and plaque rupture with thrombosis that can block blood flow acutely i.e. MI [94].

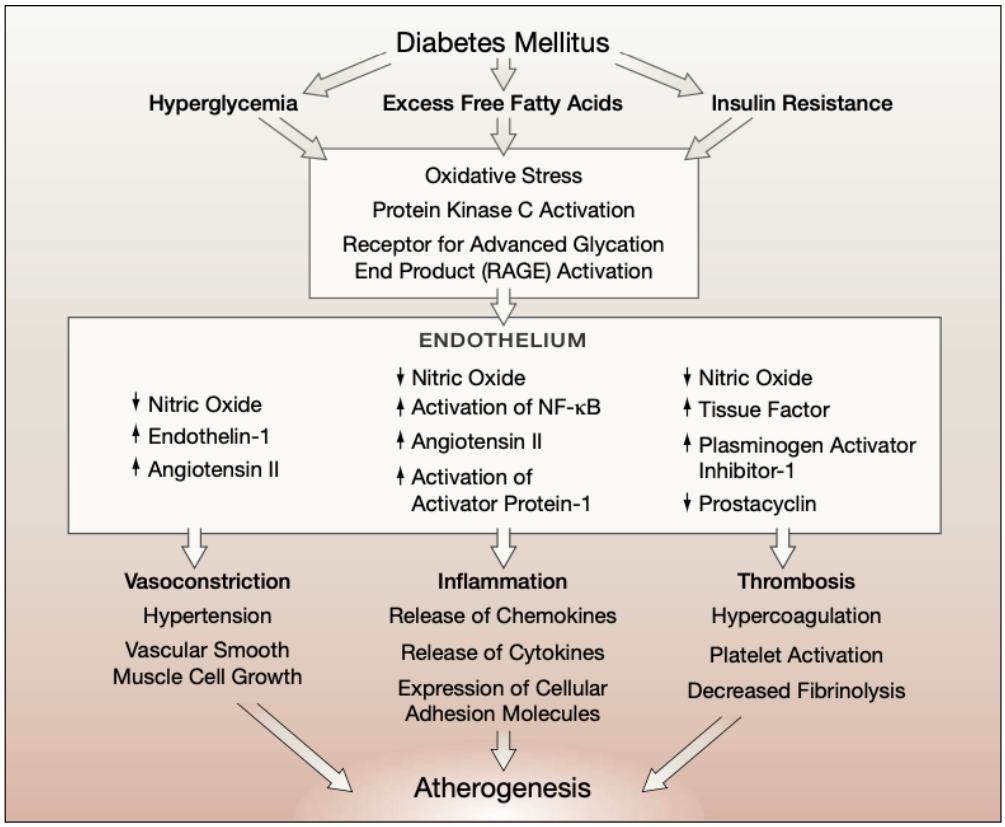


Figure 1.8 *Dysfunction in diabetes [94].*
In DM, hyperglycaemia, excess FFA release, and IR provoke adverse metabolic events within the endothelial cell. Activation of these systems impairs endothelial function, augments vasoconstriction, increases inflammation, and promotes thrombosis. Decreasing NO and increasing endothelin-1 and angiotensin II concentrations increase vascular tone and vascular SMC growth and migration. Activation of the transcription factors nuclear factor κB (NF-κB) and activator protein 1 induces inflammatory gene expression, with liberation of leukocyte-attracting chemokines, increased production of inflammatory cytokines, and augmented expression of cellular adhesion molecules. Increased production of tissue factor and plasmin activator inhibitor 1 creates a prothrombotic milieu, while decreased endothelium-derived NO and prostacyclin favours platelet activation.
Image taken from Beckman *et al.,* [94].

1.4.2 Preventing CVD

Evidence from clinical trials and observational studies have revealed that it may take years to detect the effects of glucose levels on macrovascular outcomes. In the Veteran Affairs Diabetes Trial (VADT), the 6 year long active phase of intensive glucose lowering had a neutral effect on CVD outcomes. However, a distinct and significant benefit emerged during an additional 4 years of observational follow up in people with T2DM who had been randomly assigned to the intensive glucose control group. People with T2DM in this intensive intervention group had 8.6 fewer major CVD events per 1000 person-years than those in the standard therapy group [103]. Similarly, the Epidemiology of Diabetes Interventions and Complications (EDIC) study, which followed people with T1DM for long-term complications, found intensive DM treatment reduced the 17 year incidence of CVD events by 42%, compared to conventional treatment [104], and the 27 year mortality by 33% [105]. The risk of CVD increases by approximately 20% for every 1.5 mmol/L rise in fasting glucose concentration above normal [106, 107], and for every 1% increase in HbA1c [108]. This is similar to other

risk factors associated with CVD risk e.g., 0.5 mmol/L rise in LDL-C [109], and a 5.0 mm Hg increase in systolic blood pressure [110], are both linked to a 20% increase in CVD risk. However, people with T1DM who have good glycaemic control, and no additional CVD risk factors, still have a significantly elevated risk of CVD events [111], suggesting that other factors are implicated in the elevated risk of CVD in DM.

Another effective strategy for CVD risk reduction, is management of diabetic dyslipidaemia [112], as dyslipidaemia is a key risk factor for CVD, particularly in T2DM [113]. Statins are the current gold-standard treatment for dyslipidaemia. They inhibit the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase enzyme, a key enzyme in *de novo* cholesterol synthesis [114]. This results in an upregulation in the expression of the LDL-receptor (LDLR) in the liver, which enhances LDL-C clearance from the circulation [115]. Statins are the most widely subscribed drug for cholesterol-lowering [116]. However, even among people with high statin adherence, approximately 40% do not achieve a ≤30% reduction in LDL-C, highlighting the need for other therapies and targets [117].

CVD has long been associated with chronic low-grade inflammation [84]. The Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) was the pivotal clinical study investigating the inflammatory hypothesis of atherosclerosis/ CVD, aiming to target IL-1β mediated inflammation to mitigate against the unacceptable burden of CVD events [118]. Canakinumab, a humanised monoclonal antibody, targets IL-1β without effecting lipid levels. IL-1β is a master cytokine of innate immunity, and stimulates the pro-inflammatory IL-6 signalling pathway [118]. The double-blinded CANTOS trail recruited a selective/ targeted population of 10,061 high-risk participants with prior MI, and elevated CRP levels (concentrations >2 mg/l). The drug significantly decreased the risk for the primary (MI, stroke and CVD death), and secondary end points, independent of any changes in lipid levels [118]. This indicates the potential for therapies that aim to dampen inflammation in high-risk people. Sub-analysis of the CANTOS trial revealed that canakinumab also reduced CV events similarly in those with DM, pre-diabetes and normoglycaemia [119]. However, the rate of progression to new-onset DM was not reduced during the CANTOS trial questioning the causal role of inflammation in driving the progression from IR to overt T2DM [119].

1.5 Lipoprotein metabolism

Atherosclerosis encompasses a cholesterol disorder [84]. The lipoprotein system facilitates the intercellular transport of cholesterol and TG. Lipoprotein particles are classified according to their density, ranging from chylomicrons to very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL and HDL. Each lipoprotein varies in its lipid, and protein

composition, and therefore its function. An overview of human lipoprotein metabolism is depicted in **Figure 1.9**.

1.5.1 Chylomicrons

Enterocytes via Niemann-Pick type C1 (NPC1)-like 1 (NCP1L1) absorb dietary lipids [120], after which TG, CE and other lipids are packed together with ApoB-48 to form chylomicrons. Chylomicrons are then exported to the lymph, and afterwards into the blood. The gut synthesizes ApoB-48 continuously, but lipidation to form chylomicrons is dependent on the availability of lipids, and therefore regulated by food intake. Lipoprotein lipase (LPL), which is attached to the luminal surface of endothelial cells, and found mostly in muscle, heart, and adipose tissue, hydrolyses TG, and liberates FFA into peripheral cells, especially the adipose tissue for storage, thereby performing a key role in chylomicron clearance [121]. ApoC-III can inhibit LPL [122]. The liver takes up the chylomicron remnants via the LDLR, and the LDL receptor-related protein (LRP), both of which bind ApoE on the remnant particles [121].

1.5.2 VLDL

Lipids are exported from the liver into the blood as VLDL. ApoB-100 is the main lipoprotein associated with VLDL. Like chylomicrons, LPL hydrolyses TG from VLDL in plasma, generating FFA, which can be used as fuel in the heart or skeletal muscle, or stored in adipose tissue. The progressive depletion of TG from VLDL stimulates the transfer of phospholipids (PL), ApoC and ApoE to HDL, resulting in the formation of IDL. Approximately 90% of IDL are converted into LDL via hepatic lipase; the liver clears the rest [121].

1.5.3 LDL

LDL is the primary transporter of cholesterol within the blood. It is composed of a core of CE surrounded by a shell of PL and free/ unesterified cholesterol (FC) together with a single molecule of ApoB-100. LDL uptake into cells is via receptor-mediated endocytosis. This involves the binding of LDL-ApoB-100 to the LDLR on the plasma membrane of hepatic and other tissues. The LDL-receptor complex is internalised via endocytosis, followed by fusion with lysozymes, which contain catabolic enzymes [121]. Proprotein convertase subtilisin/kexin type 9 (PCSK9) performs a central role in regulating LDLR activity. PCSK9 binds to the LDLR/ LDL-complex, targeting it for lysosomal degradation, and therefore preventing the receptor from recycling back to the surface in the liver [123].

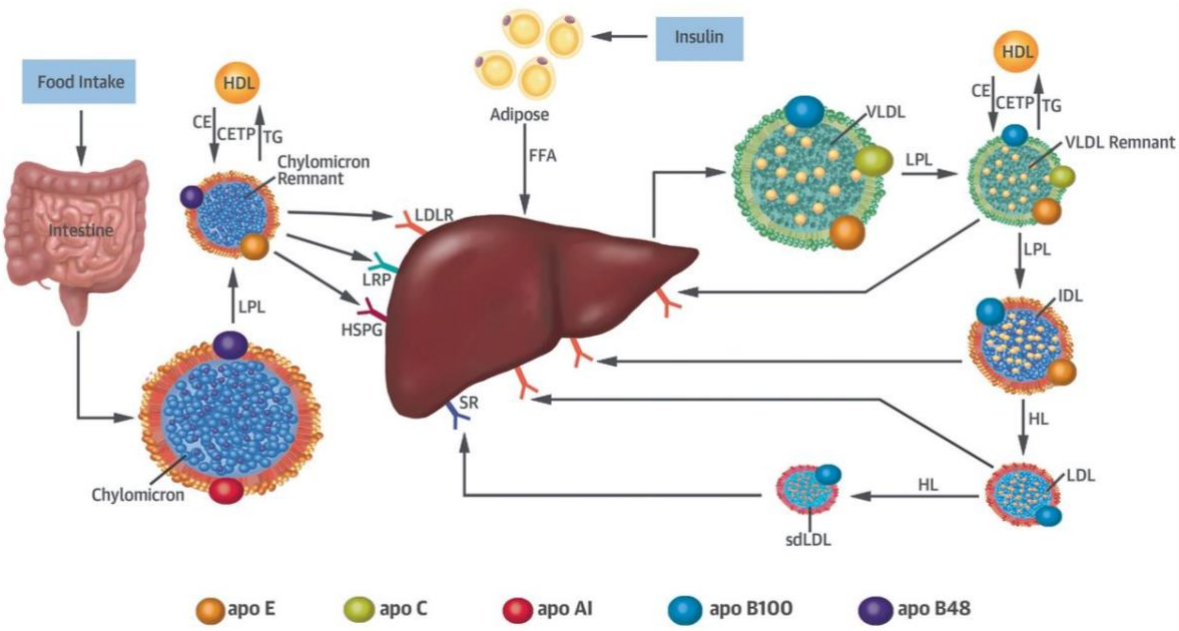


Figure 1.9 *Overview of human lipoprotein metabolism [124].*
Chylomicrons secreted from the intestine and VLDL secreted from the liver are lipolyzed by lipoprotein lipase (LPL), leading to triglyceride-rich lipoprotein (TRL) remnants. Chylomicron secretion is largely regulated by food intake, whereas VLDL secretion is controlled by insulin. Remnant particles undergo remodelling via the enzymatic action of cholesteryl ester transfer protein (CETP) with HDL, hepatic lipase, and the exchange of soluble apolipoproteins. TRL remnants are cleared from the circulation via receptor-mediated uptake involving the LDL receptor (LDLR) and LDL receptor–like protein (LRP). Chylomicron remnants and VLDL remnants compete for the same lipolytic pathway, a process mediated by ApoE. While chylomicron remnant clearance may be mediated by LDLR or LRP, VLDL remnants are believed to be predominantly cleared via LDLR.
Image taken from Saeed *et al.,* [124].

1.5.4 HDL

HDL particles (HDL-P) are a heterogenous emulsion of proteins, lipids, and microRNAs, that circulate in the blood. The biosynthesis of HDL involves the synthesis and secretion of key HDL protein components followed by the extracellular acquisition of lipid (PL and FC, in particular), which assembles to generate the mature spherical HDL-P (**Figure 1.10**).

ApoA-I, accounts for approximately 70% of the protein mass of HDL [125], and is synthesised in both the liver and the intestine [126]. Lipid efflux from cells to acceptor HDL-P can happen by mechanisms such as regulated transporter-facilitated routes, and aqueous diffusion [127]. Lipidation of the HDL apolipoproteins occur primarily after their secretion, with the ATP binding cassette subfamily A, member 1 (ABCA1), a key participant in the early acquisition of lipid to ApoA-I [126]. This lipid poor ApoA-I acquires increasing quantities of PL and FC, maturing through nascent discoidal HDL (preβ-1 HDL) to form mature spherical HDL.

The development of the mature spherical HDL necessitates the esterification of acquired FC to form cholesterol esters (CE), generating a hydrophobic lipid core within HDL. HDL-CE is formed by the action of lecithin-cholesterol acyltransferase (LCAT), a HDL-associated enzyme that catalyses the transfer of 2-acyl groups from lecithin to FC, forming CE and lysolecithin [128]. Maturing HDL-P acquire more cholesterol via interaction with the ATP-binding cassette

subfamily G, member 1 (ABCG1) and scavenger receptor class B member 1 (SR-B1) cholesterol transporters expressed on the surface of peripheral cells [129].

The kidney, liver and steroidogenic tissues are key sites of HDL catabolism. Clearance of HDL can occur by 1) selective cholesterol uptake in the liver whereby there is selective removal of cholesterol (mainly) and other lipids from the particle, without uptake of the whole particle and; 2) endocytic uptake and degradation of the whole particle in the kidney by the proximal renal tubular epithelial cells via the cubilin/ megalin system [125].

SR-B1 has high-affinity for binding to HDL, and is a major player in HDL selective lipid uptake by tissues [130]. Catabolism of all key HDL lipids is mediated by SR-B1 [131]. Lipid is transferred from the HDL core to tissues by a 2-step process of 1) binding of HDL to the receptor; and 2) diffusion of lipid into the plasma membrane without the concomitant degradation of the particle. Another method of cholesterol uptake involves cholesterol ester transfer protein (CETP), which facilitates exchange of CE on HDL for TG on ApoB-containing lipoproteins. The CE now associated with ApoB-containing lipoproteins can subsequently be taken up by the liver via the LDLR [126]. The net effect of CETP on HDL is depletion of CE and enrichment with TG [126]. There is a strong inverse link between HDL-C and TG levels in the circulation and increased TG accumulation on HDL is thought to contribute to reductions in HDL-C levels in the setting of the metabolic syndrome [132].

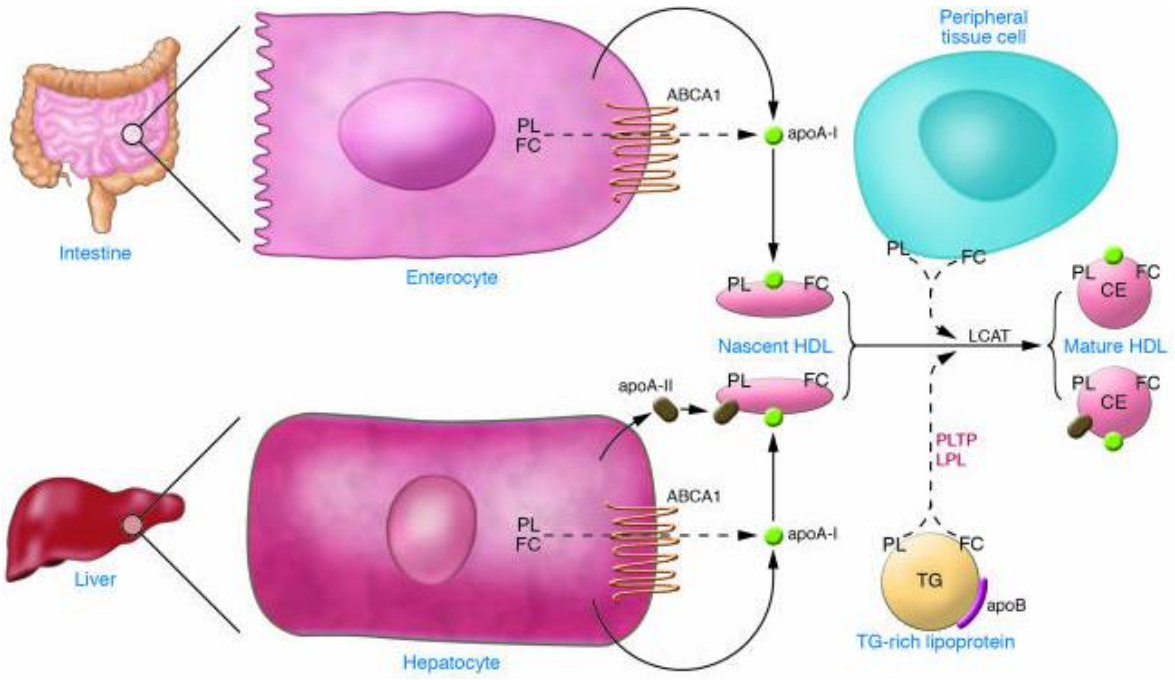


Figure 1.10 *HDL biosynthesis [126].*
Enterocytes and hepatocytes synthesize ApoA-I, which is secreted in a lipid-poor form and then immediately recruits additional PL and free cholesterol via the ABCA1 pathway, forming nascent HDL. Nascent HDL acquires more lipid from other peripheral tissues and from lipoproteins, and lecithin-cholesterol acyltransferase (LCAT) generates cholesterol esters (CE), forming mature HDL. The liver also synthesizes ApoA-II, which results in a subclass of HDL containing both ApoA-I and ApoA-II.
Image taken from Rader *et al.,* [126].

1.6 HDL particle (HDL-P) isolation

HDL is primarily isolated from serum/ plasma using either fast-protein liquid chromatography (FPLC) or density gradient ultracentrifugation (DGUC). Depending on the technique used, HDL can be isolated into different defined subclasses. DGUC, first described in 1955 [133], is considered the gold-standard for HDL isolation and therefore, is the most widespread technique used to isolate the HDL subclasses, predominately HDL_2 (density between 1.063 and 1.125 g/mL) and HDL_3 (density between 1.125 and 1.21 g/mL) [134] (**Figure 1.11**). DGUC has the advantage of quantitatively splitting the relatively light lipid-rich proteins apart from the dense non-lipoprotein associated proteins. However, speed and length of centrifugation combined with salt exposure during this process has led to questions about induced changes in HDL quality and function [135]. Additionally, the presence of copious amounts of small proteins e.g. ApoA-I, and albumin, can diminish mass spectrometry (MS) detection of the lower abundant HDL proteins due to ion suppression, and masking effects by compelling the MS instrument to devote the majority of its duty cycle to the MS/MS analysis of peptide ions derived from the highly abundant, potentially contaminant proteins [135].

The FPLC HDL isolation method separates the lipoprotein particles based on size, in the absence of salt and shear force [136]. An advantage of this method is that the HDL-P is separated into approximately 8-12 distinct subpopulations enabling analysis of the large (L-HDL), and small (S-HDL) sized HDL-P. DGUC separates based on fixed density cut-offs while FPLC utilises a continuous size distribution of particles. FPLC is a reproducible method, results in a non-destructive separation of lipoproteins, and requires less serum sample [136]. However, the primary disadvantage of separating by FPLC is the overlap in size between HDL and many high abundant plasma proteins that are assumed to be contaminants within the FPLC fractions e.g., immunoglobulins. Additionally, FPLC-isolated HDL may face a nomenclature issue as HDL has traditionally been isolated based on density, therefore, methods that separate lipoproteins based on size may lead to HDL that is not comparable.

Further techniques can be used to purify the FPLC-derived HDL and remove potential contaminants. One such method is using a hydrated calcium silicate resin (CSH), a lipid removal agent (LRA), which binds with high affinity to PL. In FPLC-HDL fractions, the dominant PL-containing particle, in turn, would be the HDL-P allowing a simple, practical and cost-effective approach to separate out HDL-associated proteins from contaminants [135]. HDL immunoprecipitation is another effective purification technique using ApoA-I affinity beads, however only proteins associated with the ApoA-I-interactome may be captured by this approach and additional HDL-associated proteins may not be detected [137]. Within this thesis, we used FPLC-isolated HDL, further purified by LRA precipitation, as our method of choice for HDL isolation for proteomics.

Lipoprotein particle concentration and sizes can also be measured using nuclear magnetic resonance (NMR) spectroscopy. Otvos *et al.,* published the first analysis on lipoproteins particles separated by NMR in 1991, based on the concept that each lipoprotein subclass emits a distinctive lipid methyl group NMR signal [138]. The amplitudes of the measured lipoprotein signals are directly proportional to the number of subclass particles. This provides the estimated mol/l concentration for the three HDL-P subclasses (large 8.9-13 nm, medium 8.3-8.8 nm, small 7.3-8.2 nm) [139].

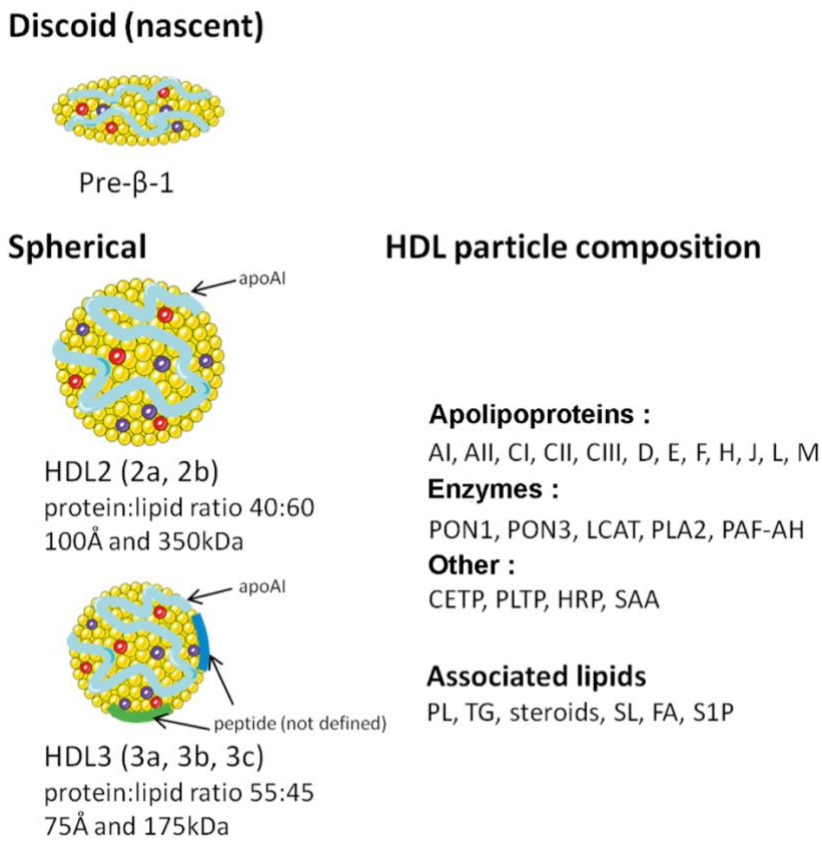


Figure 1.11 *Schematic representation of HDL particle heterogeneity [134].*
Image taken from Woudberg *et al.,* [134].

1.6.1 HDL-P size

The heterogeneity of HDL allows HDL-P to be characterised into several distinct subpopulations of varying sizes, and lipid, protein, and apolipoprotein composition. This heterogeneity is attributable to factors such as CETP and LCAT, which participate in the remodelling of the individual HDL subpopulations [126]. The cholesterol in HDL is an inert surrogate marker of the number and size of HDL-P and is unlikely to be responsible for any of the cardioprotective functions of HDL. The size of HDL-P is important in accessing their anti-atherogenic properties, with different HDL subpopulations involved in diverse biological activities (**Table *1.6*** and HDL functions expanded on in **Section 1.9**).

Multiple studies have found that higher small HDL-P (S-HDL-P) are most strongly associated with lower CVD risk [140-142]. Additionally, the Translational Research Investigating Underlying disparities in acute Myocardial infraction Patient's Health status (TRIUMP) study and the Intermountain Heart Collaborative Study (IHCS) reported that low S-HDL-P are associated with >50% higher risk of CVD [143]. However, other studies show the opposite and, by extension suggest that large HDL-P (L-HDL-P) are the more protective subclass [144, 145]. These studies vary in design, adjustment for confounders, and methods for HDL subpopulation isolation and quantification, hence, there are conflicting data concerning HDL subpopulations and subsequent prediction of CVD. Working towards resolution of this subclass controversy is ever more important given that total HDL-C levels are not necessarily predictive of risk. It is becoming increasingly apparent that measuring the size of lipoprotein particles, and not just the quantity of cholesterol carried on the particles, is another important factor of CVD risk to be considered and elucidated [146].

Table 1.6 *Biological activities of HDL subpopulations [147].*

Biological activity	Subpopulation	Feature
Cholesterol efflux	Small, dense HDL	Potent efflux from lipid-loaded macrophages Potent efflux via ABCA1 Potent LCAT activation
	Large, light HDL	Potent efflux via ABCG1 and SR-BI
Antioxidant activity	Small, dense HDL	Potent protection of LDL from oxidative damage Potent inactivation of LOOH and other oxidised PL
Anti-thrombotic	Small, dense HDL Large, light HDL	Potent anticoagulant activity Inhibition of platelet aggregation
Anti-inflammatory	Small, dense HDL	Inhibition of endothelial cells adhesion molecules expression

1.7 Diabetic Dyslipidaemia – Implications for HDL

Dyslipidaemia is a classic hallmark of obesity, and T2DM, with increased levels of small, dense LDL (sd-LDL) particles and circulating TG, and reduced levels of HDL-C [148-151]. In contrast to people with T2DM, lipid profiles in T1DM are normal or even apparently better than the general population, with greater HDL-C levels and lower LDL-C, and TG levels [152].

Insulin plays a central role in lipoprotein metabolism (**Figure 1.12**); therefore, it is not surprising that derangements in insulin function contribute to dyslipidaemia. Insulin is a potent stimulator of LPL, promoting the catabolism of triglyceride-rich lipoproteins (TRL) and facilitating storage of fatty acids in adipose tissue. Insulin inhibits the expression of ApoC-III, an inhibitor of LPL [153]. Insulin also enhances LDL-R expression and activity, which drives the clearance of LDL-C from circulation [154].

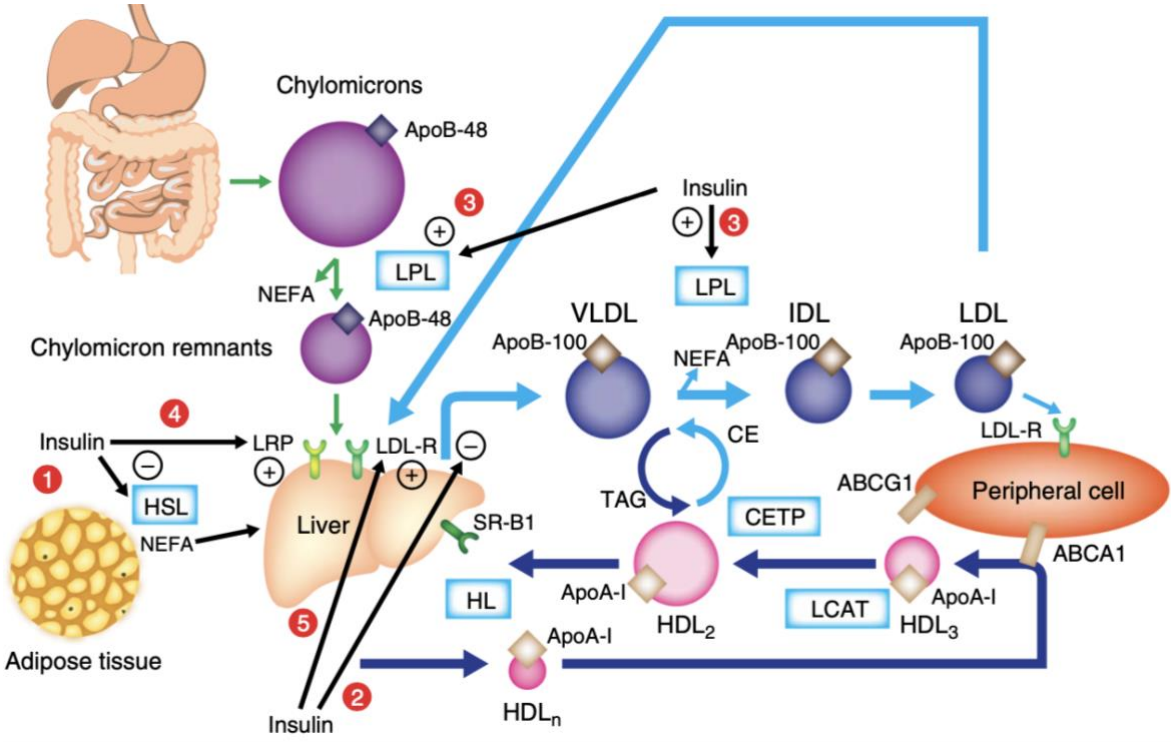


Figure 1.12 *Overview of human lipoprotein metabolism, and the effects of insulin [121].*
(1) Insulin inhibits hormone-sensitive lipase. (2) Insulin inhibits hepatic VLDL production. (3) Insulin activates LPL. (4) Insulin increases LRP expression on the plasma membrane. (5) Insulin increases LDL-R expression. Image taken from Vergès *et al.,* [121].

1.7.1 Dyslipidaemia in T2DM

The lipid abnormalities associated with T2DM are not only quantitative, but also qualitative and kinetic [121] (**Table *1.7***, and **Figure 1.13**).

T2DM has an important effect on HDL such that an accumulation of TRL, secondary to LPL deficiency, increases CETP-mediated lipid exchange between TRL and HDL, thereby enriching HDL-P with TG and depleting them of CE [155]. This results in lower plasma HDL-C concentrations as TG-enriched HDL lipids are predisposed to phospholipid transfer protein (PLTP)-mediated PL exchange, and hepatic lipase-mediated clearance of HDL from the circulation and therefore, increased catabolism [125].

Table 1.7 *Key changes in lipoprotein metabolism in T2DM [121].*

Lipoprotein	Quantitative changes	Qualitative changes	Kinetic/ metabolic changes
VLDL	↑ plasma concentration	↑ proportion of larger particles ($VLDL_1$); ↑ palmitic acid-containing species and diacylglycerol, reduced sphingomyelin; Glycation	↑ production; ↓ catabolism

LDL	No change or slightly ↑ plasma concentration	↑ proportion of small, dense particles (TG enrichment); ↑ LDL oxidation; ↑ palmitic acid-containing species and diacylglycerol, reduced sphingomyelin; Glycation	↓ catabolism
HDL	↓ plasma concentration	TG enrichment; ↓ PL, ApoE and ApoM; Glycation	↑ catabolism

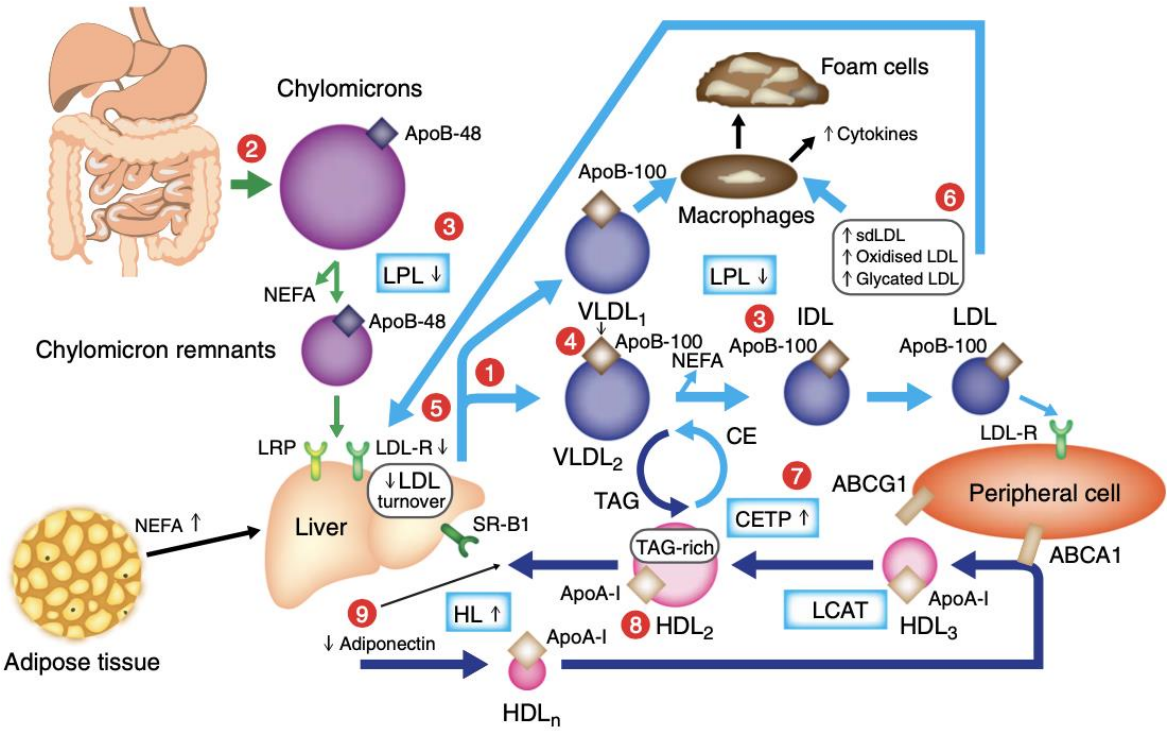


Figure 1.13 *Main lipid abnormalities in T2DM [121].*
(1) increased VLDL production (mostly $VLDL_1$) , (2) increased chylomicron production, (3) reduced catabolism of both chylomicrons and VLDL (diminished LPL activity), (4) increased production of large VLDL ($VLDL_1$), preferentially taken up by macrophages; LDL (qualitative and kinetic abnormalities): (5) reduced LDL turnover (decreased LDL-R), (6) increased number of glycated LDL, small, dense LDL (TG-rich) and oxidised LDL, which are preferentially taken up by macrophages; HDL (low HDL-cholesterol, qualitative and kinetic abnormalities): (7) increased CETP activity (increased transfer of TG from TG-rich lipoproteins to LDL and HDL), (8) increased TG content of HDL, promoting HL activity and HDL catabolism, (9) low plasma adiponectin favouring the increase in HDL catabolism.
Image taken from Vergès *et al.,* [121].

1.7.2 Dyslipidaemia in T1DM

The effect of T1DM on the lipoprotein profile is remarkably different depending on how poorly or well controlled hyperglycaemia is. In the Coronary Artery Calcification in Type 1 Diabetes (CACTI) study, each 1% increase of HbA1c was associated with an elevation in LDL-C, non-HDL-C, and TG of 0.103 mmol/l, 0.129 mmol/l, and 0.052 mmol/l, respectively [156]. This

signifies that hyperglycaemia is a key factor leading to quantitative lipid abnormalities in T1DM (**Figure 1.14**).

People with well-controlled T1DM, whereby insulin is repleted with exogenous insulin, show normal or decreased plasma TG, cholesterol, and LDL-C levels, and normal or increased HDL-C levels [157, 158], highlighting that exogenous insulin is sufficient to restore lipoprotein homeostasis. The increased plasma HDL-C levels could be the outcome of the elevated LPL/ hepatic lipase ratio observed in people with well-controlled T1DM [159].

However, this 'normal' lipoprotein profile may mask subtle changes to the quality of each of the lipoprotein particles. HDL abnormalities in T1DM include TG enrichment of HDL due to increased CETP activity [160]; glycation of ApoA-I, which can impair the HDL mediated reverse cholesterol transport (RCT) pathway [161]; increased oxidation [162]; and changes in the protein and lipid composition of the HDL-P.

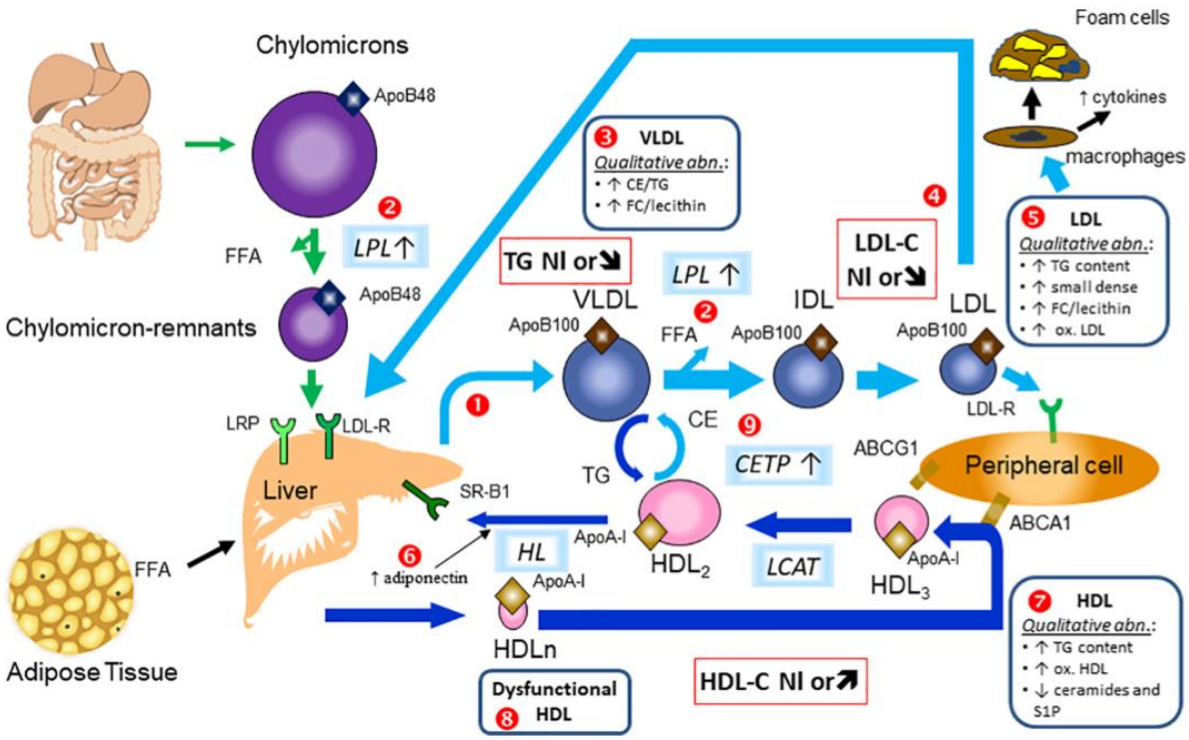


Figure 1.14 *Main lipid abnormalities in T1DM with optimal glycaemic control [163].*
TG (plasma level normal or frequently reduced; qualitative and kinetic abnormalities): (1) reduced VLDL production; (2) increased catabolism of both chylomicrons and VLDL (increased LPL activity); (3) VLDL qualitative abnormalities: increased CE/TG ratio, increased free cholesterol/lecithin ratio. LDL abnormalities (plasma level normal or reduced; qualitative and kinetic abnormalities): (4) increased LDL catabolism; (5) qualitative abnormalities: increased number of small dense LDL (TG-rich) and oxidized LDL, which are preferentially taken up by macrophages, increased free cholesterol/ lecithin ratio. HDL abnormalities (HDL-C normal or frequently increased, qualitative and kinetic abnormalities): (6) reduced HDL catabolism; (7) qualitative abnormalities of HDL: increased TG content, increased oxidation of HDL, reduced ceramides and S1P; (8) dysfunctional HDL; (9) increased CETP activity (increased transfer of TG from TG-rich lipoproteins to LDL and HDL).
Image taken from Vergès *et al.,* [163].

1.8 HDL raising therapies

HDL-C has been shown to be a robust, consistent, and independent predictor of CVD [164, 165]. Other studies have shown that raising HDL-C by 1 mg/dL was linked to a 2-4.7% decreased risk of CHD [166]. In addition, the Emerging Risk Factors Collaboration study (*n*=302,430) found that the hazard ratio (HR) for CHD with HDL-C was 0.71 after adjustment for non-lipid risk factors [109]. These observations instigated the development of therapies that would raise HDL.

1.8.1 CETP inhibitors

Humans with a genetic CETP deficiency have increased levels of HDL-C combined with lower levels of LDL-C [167]. These observations instigated the development of CETP inhibitors. The pharmaceutical industry heavily invested in these therapies, and conducted several clinical trials, with thousands of participants, to evaluate their effectiveness (**Table 1.8**). CETP inhibitors block the rate of transfer of TG from TRL to HDL in exchange for CE. This in turn increases the CE content of HDL, forming larger HDL-P that are slower to catabolise [168].

CETP inhibitors very successfully raised HDL-C but failed to deliver an anti-atherogenic effect. The first large trial with torcetrapib, Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) was terminated early due to increased mortality, and increased risk of CVD in people subscribed the inhibitor. Potential causes of increased mortality were thought to be unanticipated side effects of CETP inhibition, particularly hypertension [169]. The Effects of the Cholesterol Ester Transfer Protein Inhibitor Dalcetrapib in Patients with Recent Acute Coronary Syndrome (dal-OUTCOMES) and the Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition with Evacetrapib in Patients with a High Risk for Vascular Outcomes (ACCELERATE) trial were also both terminated early due to lack of efficacy [170, 171]. The Randomized Evaluation of the Effects of Anacetrapib through Lipid modification (REVEAL) trial found that CVD events were reduced in people who took Anacetrapib (HR=0.91) [172]. Possible reasons for why anacetrapib succeeded, compared to the other CETP inhibitors which failed, are relative safety (compared to torcetrapib), potency (compared with dalcetrapib), a study design with adequate statistical power (largest number of participants compared to all trials), and sufficiently long duration to uncover the benefit (compared to evacetrapib) [168]. However, Merck did not bring forward anacetrapib for FDA approval, concluding that ‘the clinical profile for anacetrapib does not support regulatory filings’ – Roger Perlmeutter MD, PhD, president of Merck Research Laboratories. While the drug showed a small favourable effect, it was probably not large enough to make it a viable therapy. The safety concerns included a slight increase in blood

pressure (+0.7 mmHg), and a prolonged half-life with the drug accumulating within the adipose tissue for several years despite cessation [173].

Table 1.8 *Clinical trials investigating CETP inhibitors.*

Trial	**ILLUMINATE [169]**	**dal-OUTCOMES [170]**	**ACCELERATE [171]**	**REVEAL [172]**
Drug	*Torcetrapib*	*Dalcetrapib*	*Evacetrapib*	*Anacetrapib*
Pharmaceutical company	Pfizer, NY, USA	Hoffmann-La Roche, Basel, Switzerland	Eli Lilly & Company, IN, USA	Merck, NJ, USA
Sample size	*n* = 15,067	*n* = 15,871	*n* = 12,092	*n* = 30,449
Population characteristics	45-75 yrs old; Diabetes or history of CVD 30 days to 5 yrs before screening	≥45 yrs old; Post-acute coronary syndrome	≥18 yrs old; High CVD risk	≥50 years old; History of CVD
Trial design	Placebo-controlled; Randomized; Double-blinded	Placebo-controlled; Randomized; Single-blinded	Placebo-controlled; Randomized; Double-blinded	Placebo-controlled; Randomized; Double-blinded
Duration	Median of 18 months follow-up	Median of 31 months follow up	Terminated at 26 months	4.1 years follow-up time
HDL % increase	72%	31-40%	133%	104%
Outcome in CETP inhibitor group	↑ risk of CVD events (HR = 1.25); ↑ risk of death from any cause (HR = 1.58); ↑ BP (+5.4 mmHg)	↑ CRP; Terminated on futility: No reduction in recurrent CVD events; ↑ BP (+0.6 mmHg)	No lowering of CVD events; Lack of efficacy ↑ BP (+1.2 mmHg)	↓ CVD events (HR = 0.91); ↓ new-onset diabetes; Small ↑ BP (+0.7 mmHg); No difference in deaths

1.8.2 Niacin or nicotinic acid

Niacin or nicotinic acid is an efficient HDL-C raising drug. When administered daily in doses of 1-3g, it usually raises HDL-C by 15-40% [174]. Its mechanisms of action include non-competitive inhibition of hepatocyte microsomal diacylglycerol acyltransferase-2, an enzyme which catalyses the final step in TG synthesis [175], as well as selective inhibition of ApoA-I uptake without affecting *de novo* synthesis [176]. However, adverse side-effects have hampered the widespread use of niacin treatment, in particular, vasocutaneous flushing.

The Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides and Impact on Global Health Outcomes (AIM-HIGH) trial, which enrolled 3,414 statin-treated individuals with established CVD and low baseline levels of HDL-C, failed to demonstrate a clinical benefit of extended-release niacin (ERN) over statin therapy alone [177]. While, ERN therapy elevated HDL-C from 35 mg/dL to 42 mg/dL and reduced TG from 164 mg/dL to 122 mg/dL at the two-year point, the trial was terminated after 3 years of follow-up due to lack of efficacy. Another trial, The Heart Protection Study 2 – Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) recruited 25,673 statin-treated high-risk individuals with prior vascular disease to assess the effects of adding ERN in combination with laropiprant, a drug that reduces flushing, on CVD outcomes [178]. The ERN–laropiprant-treated group displayed reductions in LDL-C (−10 mg/dL) and increases in HDL-C (+6 mg/dL) compared with placebo but reported no improvements in cardiovascular events, similar to the AIM-HIGH trial outcomes. Additionally, ERN–laropiprant treatment was associated with significantly more adverse events including increased diagnosis of DM.

1.8.3 HDL infusion therapies

HDL infusion therapies include isolated, partially delipidated HDL proteins, native ApoA-I or genetic variants such as ApoA-I Milano complexed with phospholipids [179]. Recombinant HDL infusion agents not only rapidly raise the number of circulating HDL-P, but their ApoA-I can also be reconstituted with a variety of different phospholipids that have the potential to impart specific biological actions [179]. Small scale studies (*n* ranging from 7-57 subjects) have shown that HDL infusions can induce a rapid, dose-proportional and time-dependent elevation in ApoA-I and preβ-1 HDL-P, and enhance cholesterol efflux [180-183]. This has led to the suggestion that HDL infusion therapies have potential atheroprotective effects, which could provide a novel option to rapidly lower the systemic burden of atherosclerosis. However, the half-life of ApoA-I in these treatments is approximately 48-72 hours, so the effect duration is relatively short. Additionally, a high amount of infused HDL, comparable in magnitude to the total circulating pool of ApoA-I, seems to be needed for clinical efficacy. Larger studies paint a more controversial picture, with administration of an engineered preβ-1 HDL-P having no detectable effect on atherosclerotic plaque composition or regression compared to placebo [184].

1.8.4 Looking beyond HDL-raising therapies

The failure of HDL raising therapies is compounded by evidence from Genome Wide Associations Studies (GWAS), which suggest that genetically low HDL-C levels are not a determinant of CVD [185-187]. This is in stark contrast to epidemiological studies

demonstrating the opposite, highlighting the disconnect between genetically altered HDL-C levels, and epidemiological observations of HDL-C. The HDL hypothesis of increasing HDL-C to decrease risk of CVD seemed to be disproved. However, there is the possibility that measurements of static HDL-C are an oversimplified estimate of HDL biology. A simple measurement of HDL-C levels may mask subtle changes within the HDL-P that influence its function. The diversity and complexity of HDL-P gives rise to emerging distinct functions for HDL [125] in areas as diverse as lipid transport, oxidation, inflammation and immunity [188]. However, the relationship between HDL composition and function is poorly understood and was a major area of focus of this thesis.

1.9 HDL functionality

1.9.1 Cholesterol efflux capacity (CEC)/ Reverse cholesterol transport (RCT)

RCT is the cardioprotective pathway within which HDL accepts cholesterol from peripheral cells such as adipocytes, endothelial cells, and macrophages (particularly lipid-laden foam cells in atherosclerosis) for delivery to the liver and subsequent elimination in the bile and faeces [129, 189-191] (**Figure 1.15**).

The first step in the macrophage-to-faeces RCT pathway plays a key role in maintaining intracellular cholesterol homeostasis. Cholesterol is effluxed from macrophages via the cholesterol transporters ABCA1, ABCG1 transporters and SR-B1 [129, 192-195]. ABCA1, expressed on both adipocytes and macrophages, is responsible for efflux to the ApoA-I protein, associated with lipid-free/ lipid-poor HDL-P [196, 197], while ABCG1 expressed on macrophages, but not adipocytes, promotes efflux to larger, mature HDL-P [129, 189]. SR-B1 is a bi-directional cholesterol transporter that can also interact with mature HDL-P expressed on both cell types [130, 189]. Macrophage cholesterol efflux contributes minimally to overall flux through the RCT but it is arguably the most crucial in mitigating the development of atherosclerosis [198].

The final stage of the RCT pathway is where HDL-P loaded with cholesterol travel to the liver where the FC and CE are selectively taken up from the HDL-P via SR-B1 expressed on hepatocytes [199]. Once acquired by the liver, CE must be hydrolysed to FC. FC can be directly exported into the gallbladder via ABCG5/8 or converted into bile acids via the cholesterol 7α-hydroxylase (CYP7A1) enzyme, before export to the gallbladder via ABCB11 [200, 201]. While it is clear that SR-B1 is key for hepatic cholesterol clearance, its bidirectional flux abilities within macrophages seem to highlight that net cholesterol efflux via SR-B1 is trivial compared to ABCA1 and ABCG1 efflux [202].

The role of HDL in cholesterol efflux is frequently measured *ex vivo* using HDL-, ApoA-I- or ApoB-depleted serum isolated from both humans and animals as acceptors for cholesterol-

loaded cells [203, 204]. This model has been used to demonstrate that CEC is independent of HDL-C, and that it is the concentration of preβ-1 HDL that affects CEC [205]. Several studies using 'cholesterol efflux assays' have shown that HDL-CEC function is a stronger predictor of CVD than HDL-C levels alone [206-208]. These studies indicate that the capability of serum HDL-C to remove cholesterol from peripheral cells is a promising metric for assessing the cardioprotective properties of HDL, and that the functionality of HDL is more important than absolute HDL-C levels in predicting CVD risk. However, the JUPITER trial (Justification for the Use of Statins in Prevention: An Intervention Trail Evaluating Rosuvastatin), showed that serum HDL-CEC was not related to incidence of CVD (MI, unstable angina, arterial revascularisation, stroke or CVD death) in subjects (*n*=1050) at baseline [209]. Instead, HDL-P number, measured by NMR was the strongest predictor out of four HDL-related biomarkers (CEC, HDL-C levels, ApoA-I and HDL-P number) to show an inverse association of incident CVD events. Therefore, there is still ambiguity over whether serum HDL-CEC or measures of HDL-P size is the best predictor of CVD. The Multi-Ethnic Study of Atherosclerosis found that higher CEC level was significantly associated with lower odds of CVD (odds ratio (OR), 0.82 per SD of CEC, *n*=465). A subgroup analysis showed higher CEC was associated with a lower risk of incident CHD (OR, 0.72 per SD of CEC) but no association was found with stroke. Another cohort within this study found that higher CEC was significantly associated with higher odds of carotid plaque progression (OR, 1.24 per SD of CEC, *n*=407) [210]. The different study endpoints of CVD being used, both within and between studies, are likely influencing the discrepancies of the results. It's also important to note that HDL-CEC assays are not standardized, making it difficult to compare studies and ascertain whether *ex vivo* experiments translate to clinically relevant endpoints given their complexity and variability.

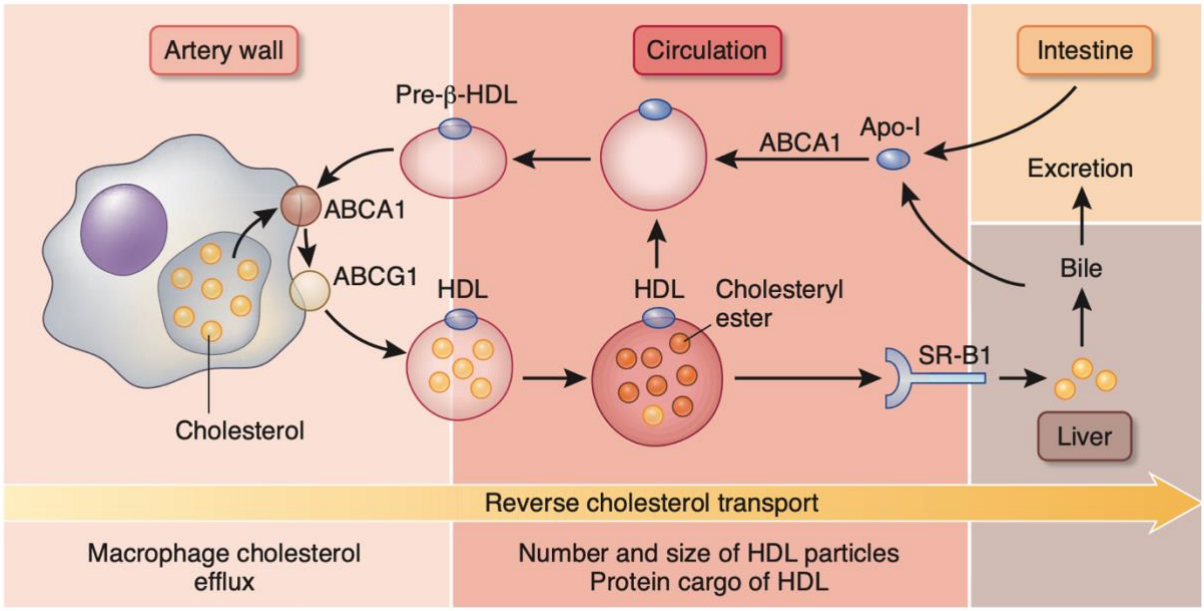


Figure 1.15 *Overview of reverse cholesterol transport by HDL [211].*
Enterocytes and hepatocytes synthesize ApoA-I. Lipid-free or lipid-poor preβ-HDL containing ApoA-I is the initial acceptor of the cholesterol exported via ABCA1 on macrophages. ABCG1 promotes efflux to larger, mature HDL particles. The enzyme lecithin-cholesterol acyltransferase (LCAT) converts free cholesterol in nascent HDL to cholesterol esters (CE), forming mature HDL. HDL that has accepted cholesterol from artery-wall macrophages re-enters the circulation, where it transports cholesterol back to the liver for excretion in the bile. CE is removed from HDL by a membrane protein on hepatocytes, SR-B1. CE is converted back to cholesterol and then bile acids, which are secreted into the bile for excretion.
Image taken from Heinecke *et al.,* [211].

1.9.2 HDL antioxidative functions

ApoA-I is multifunctional, as it is not only involved in CEC, but also plays a role in preventing aortic endothelial cells from oxidising LDL [212]. Additionally, methionine residues on ApoA-I, play a central role in HDL-mediated antioxidative activity, as they have the ability to decrease LDL-associated lipid hydroperoxides (LOOHs), the principal products of free radicals, to inactive lipid hydroxides (LOHs), thereby terminating chain reactions of lipid peroxidation [213]. Small, dense HDL can inhibit oxidation more effectively than large HDL [214]. This highlights the possibility that treatments which raise the levels of large HDL may not improve the antioxidant functions of HDL.

The primary antioxidant protein associated with HDL is paraoxonase 1 (PON1), which has been shown to hydrolyse short-chain oxidised PL on LDL, thereby protecting against oxLDL and atherosclerosis [215, 216]. However, another study has shown that hydrolysis of oxidized PL by HDL is facilitated by platelet activating factor acethyldrolase (PAF-AH) as opposed to PON1 [217, 218]. This highlights the complexity of unravelling HDL-associated proteins from assigned functions of the particles and remains a major challenge for the HDL field.

The antioxidative function of HDL can be independent of its role in CEC. However, the two roles can intertwine. The binding of ApoA-I to ABCA1 or reconstituted HDL (rHDL) to ABCG1 and SR-B1 has been demonstrated to increase the expression of superoxide dismutase, an

antioxidative enzyme, in human-monocyte-derived macrophages, while concurrently decreasing expression of Nox2 under oxidative stress conditions [219]. HDL maintains endothelial cell function by driving the ABCG1-dependent efflux of 7-ketocholesterol (7-KC), an oxysterol that is enriched on oxLDL. This results in decreased 7-KC-mediated ROS generation in human aortic endothelial cells (HAECs) and the rescue of eNOS activity, a marker of endothelial cell function [220]. LCAT, also has an antioxidative role, and can hydrolyse oxidised PLs, although probably less effectively that PAF-AH [221, 222].

1.9.3 HDL anti-thrombotic functions

Another important function of HDL is the ability to attenuate thrombosis. In particular, HDL can stimulate prostacyclin (PGI_2) release from endothelial cells [223]. PGI_2, derived from arachidonic acid, is a potent inhibitor of platelet activation [224], promotes smooth muscle relaxation and decreases the secretion of growth factors that drive vascular SMC proliferation [224]. Other actions of PGI_2 include inhibition of platelet aggregation and adhesion, inhibition of leukocyte activation and adhesion, and reduction of cholesteryl ester accumulation in vessel wall cells. These biological actions of PGI_2 suggest that it is an endogenous antiatherogenic molecule [225] that is induced by HDL-P.

In addition, HDL limits vasodilation through modulation of eNOS. Mechanistically, HDL stimulates eNOS activity via SR-B1 [226] and sphingosine 1-phosphate (S1P) receptors 1 and 3 [227, 228]. HDL induces Akt phosphorylation, extracellular signal-regulated kinase (ERK) 1/2, and intracellular calcium ion release, which play roles in a sequence of activation steps leading to phosphorylation of eNOS at Ser-1177 [227, 229]. eNOS converts L-arginine to L-citrulline with NO being produced. NO decreases vascular smooth muscle tone and reactivity [230], thus it is vasoprotective.

1.9.4 HDL anti-inflammatory functions

The beneficial effects of HDL on cardiometabolic disease are also postulated to be mediated through its multiple anti-inflammatory effects, some of which are dependent on its antioxidative effects. Monocyte chemoattractant protein-1 (MCP-1) is involved in the recruitment of monocytes and therefore, plays a key role in the inflammatory apparatus of atherosclerosis [231, 232]. MCP-1 expression is stimulated by oxLDL. HDL has been shown to inhibit the production of MCP-1 in vSMC as well as reduce ROS production and NAD(P)H-oxidase activation. Lysosphingolipids on the HDL-P facilitated these functions and the process is dependent on SR-B1 [233]. Additionally, PON1 inhibits oxLDL-induced generation of MCP-1 by endothelial cells *in vitro* [234]. This highlights that the direct antioxidative effects of HDL on oxLDL drive the anti-inflammatory benefits and the two functions are inherently linked.

One of the primary anti-inflammatory characteristics of HDL is understood to be its ability to sequester lipopolysaccharide (LPS) in circulation rendering it incapable of activating the toll-like receptor 4 (TLR-4) signalling pathway in monocytes [235, 236]. Surface PL on rHDL have been demonstrated to bind and sequester LPS in human whole blood [237]. These effects have translated from *ex vivo* experiments to *in vivo* tests with raised HDL levels protecting against an LPS challenge in mice [238], and infusion of rHDL significantly decreasing proinflammatory cytokine secretion following an inducement of an LPS insult in humans [239]. Indeed, low levels of HDL-C are prognostic of poor outcomes in people with sepsis [240], and have also been associated with poorer outcomes in people with COVID-19 disease [241].

Activated endothelial cells express VCAM-1, ICAM-1, and E-selectin, which drives the adhesion of leukocytes to the blood vessel walls. The pro-inflammatory nuclear factor-kappaB (NF-κB) signalling pathway regulates the activation of endothelial cells [242]. Pre-incubation of human umbilical vein endothelial cells (HUVECs) with HDL decreases TNFα -induced expression of VCAM-1, ICAM-1, and E-selectin [243, 244]. This anti-inflammatory property is predominately associated with smaller, dense HDL, and is independent of ApoA-I as determined by replacement of ApoA-I with ApoA-II [244].

Monocytes start differentiating into pro-inflammatory M1 macrophages after migration in the endothelial monolayer, subsequently accelerating atherosclerosis. In the presence of HDL, human blood monocyte differentiation into M1 macrophages is inhibited *ex vivo* [245]. Reduced T-cell-induced secretion of cytokines such as TNFα, IL-1β, and IL-6 occurred after addition of HDL to co-cultures of human peripheral blood monocytes and activated T-cells, dampening the pro-inflammatory response of the monocytes [246].

The sequestering and decreasing bioavailability of the inflammatory protein serum amyloid A (SAA) in circulation may also be an important anti-inflammatory effect of HDL. This can decrease SAA-induced secretion of IL-6 and MCP-1 from macrophages along with decreasing expression of proinflammatory factors [247-249]. However, it is noteworthy that accumulation of SAA on HDL can displace ApoA-I, resulting in reduced ability to inhibit MCP-1 expression in vSMCs [250]. Finally, the direct ability of HDL to stimulate cholesterol efflux from macrophages, is associated with diminished TLR4-mediated macrophage inflammatory response, thereby connecting the CEC of HDL to its anti-inflammatory functions [251, 252].

1.10 HDL composition – implications for functionality

HDL is a heterogenous complex emulsion of lipids, proteins and microRNAs, and changes in the composition of any of these individual components may influence particle functionality.

HDL has a high protein to lipid ratio whereby ApoA-I predominates, comprising 70% of the total protein mass of the particle [125]. Over 150 lipids [253], 304 microRNAs (miR) [137], and

251 proteins [254] have been shown to be associated with HDL. This variety of lipids, miR and proteins add to HDL-P diversity and dispersity, and therefore makes it improbable that the identical profile of lipids, miR and proteins exists on each HDL-P [134]. Advancements in omic technologies have given us great insight into the complexity of HDL-P and argues that there is a continuum within HDL-P diversity. Much work is now needed to understand the contribution of sub-particle populations to mediating the cardioprotective properties of HDL, and in turn the consequence of changes in HDL composition on functional properties.

1.10.1 HDL Lipidome

Over 150 different lipids have been identified on HDL-P [253]. FC accounts for 5-10% of total HDL lipid content, and is dispersed throughout the surface layer of the particle [253, 255]. Most of the sterol constituent of the HDL lipidome comprises of CE at 30-40%, which are formed through an enzymatic reaction of LCAT with FC [255, 256]. CE are more hydrophobic and therefore, congregate in the core of the HDL-P [256]. PL are the primary constituents of the HDL lipidome comprising between 40-60% [255]. The most abundant PL are phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and phosphatidylethanolamine (PE) and contribute to HDL structure, and fluidity of the surface lipid monolayer [253]. Other lesser abundant PL include the negatively charged phosphatidylinositol and phosphatidylserine which contribute to the surface charge of HDL and therefore are involved with HDL interactions with HL [257]. The HDL lipidome also has a functional role in cholesterol efflux. PC enrichment enhances SR-B1-dependent cholesterol efflux, while sphingomyelin (SM) enrichment inhibits SR-B1-dependent influx of HDL cholesterol *in vitro* [258]. Another HDL sphingolipid, S1P, has been shown to be involved in preventing ischemic injury, reducing cytotoxicity, inducing prostacyclin release, and preventing LDL oxidation [134].

1.10.2 HDL Associated microRNAs (miR)

The classic view of HDL is that it is a delivery vehicle for the return of excess cellular cholesterol from the circulation to the liver for removal. However, it has been recently shown that HDL-P from human plasma contain and deliver miR [137]. This role for HDL potentially elevates lipoproteins to mediators of systemic gene expression adding more complexity to the already diverse particle.

Vickers *et al.* were the first to show that human HDL-P contain miR, which can subsequently be delivered *in vitro* to hepatocytes with functional targeting capabilities, resulting in altered gene expression. This interaction was found to be dependent on SR-B1 and shows a novel mechanism of HDL-to-cell communication [137]. This study also revealed the differential miR profiles associated with HDL in healthy participants versus in people with familial

hypercholesterolaemia (FH), with the FH HDL-miR profile enriched with miR-223, which increased 3,780.6 fold, while miR-135a* was the most abundant miRNA associated with heathy participants [137]. These findings indicate that HDL-miR cargo may be a novel biomarker for cardiometabolic disease, however in our experience isolation of sufficient quantitates of miR from HDL is challenging.

1.10.3 HDL Proteomics

The number of HDL-associated proteins reported ranges from 9 to nearly 500 [259]. This reflects differences in the HDL isolation method, the sensitivity and type of mass spectrometry used to detect individual proteins, and the presence or absence of disease [259]. Proteomic analyses of HDL have provided a consensus view that 251 proteins are reliably associated with HDL as validated in three separate reports from three independent laboratories [254]. The true complexity of the HDL proteome has only been appreciated in the last few years, with the developmental advances of 'omic' technologies.

One of the earliest HDL proteomics studies, confirmed the presence of ApoA-I, ApoA-II, ApoM, ApoC-III, ApoC-II, SAA-IV, SAA, ApoC-I, alpha-amylase salivary, ApoL, and alpha-1-antitrypsin on HDL. It was also one of the first to highlight possible differences in the proteomic composition between different sized particles [260]. Since then it has been shown that L-HDL-P are enriched with LCAT, PLTP, and ApoE, while SAA1, alpha-1-antitrypsin and vitamin D binding protein are enriched on S-HDL-P [261], highlighting that the different HDL subpopulations have distinct compositions.

A landmark study in 2007 was conducted using shotgun proteomics to identify HDL-associated proteins in control and CAD participants. 48 proteins were identified on total HDL and small HDL_3 (S-HDL-P) isolated by UC. Of these, 22 of the HDL-associated proteins were linked to cholesterol and lipid metabolism, 23 were acute phase response proteins, 8 proteins were endopeptidase inhibitors, and the other 6 proteins were regulators of complement activations (**Figure 1.16**). The association of numerous proteins involved in the acute-phase response supports the role of HDL in the inflammatory response. ApoC-IV, PON1, complement C3, ApoA-IV and ApoE were all significantly enriched on the smaller HDL_3 particles from people with CAD [262]. Differences in the HDL_3 proteomic profile from people with CAD compared to controls suggest that potential alterations could impact on the quality of HDL and therefore HDL functionality in people with CAD.

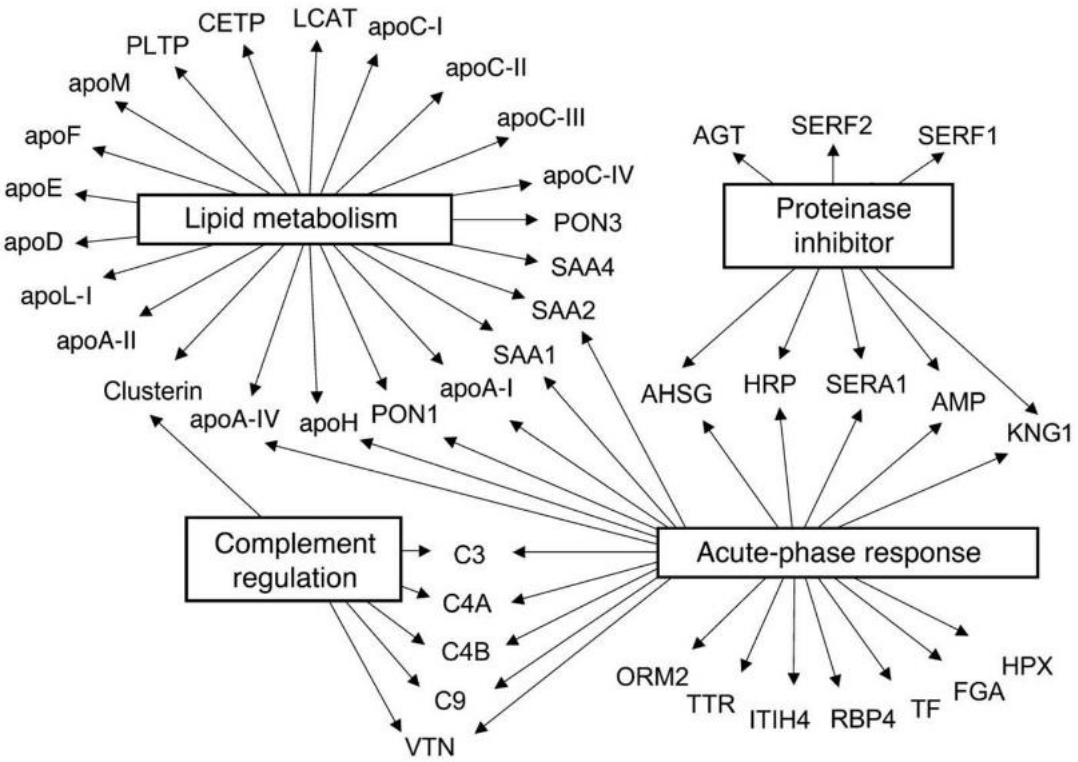


Figure 1.16 *The early proteomic diversity of HDL [262].*
Global view of biological processes and molecular functions of HDL proteins. Proteins detected in HDL were associated with biological functions using GO process annotations. This approach demonstrated significant overrepresentation of proteins involved in several categories, including lipid metabolism, the acute-phase response, protease inhibitor activity, and complement regulation.
Image taken from Vaisar *et al.,* [262].

Other studies have found that the HDL proteome is enriched with pro-inflammatory proteins in CVD as well as in T2DM, which may alter cardioprotective functions [162, 263]. However, whether changes in HDL proteomic profiles are indicative of casual or consequential deviations in CAD are unanswered, and are important future avenues to explore for pharmacological strategies and therapies. The proteins identified on HDL generally align with their assigned functions which highlights that HDL-P play an active role in protecting against atherosclerosis, and that changes in HDL composition could significantly impact HDL functionality. This suggests that the HDL proteome may proffer a novel clinically applicable tool to direct clinical decision making and better management of people at high risk of CVD, such as those with obesity and/or DM. However, it is critical that we gain a greater understanding about the relationship between HDL-proteins and HDL function in order to identify the key proteins to use within novel biomarker panels for CVD risk assessment.

Within this thesis we investigated the relationship between HDL proteomic composition and HDL efflux capacity to gain greater insight into the structure-function relationship of HDL. We further investigated whether HDL function and proteomic composition are modulated in metabolic diseases including obesity, T2DM and T1DM to further understand the residual CVD risk present in these people beyond the non-obese population.

Thesis Objective:

The main objective of this thesis was to gain greater understanding of HDL-P biology, and in particular the relationship between HDL protein composition and HDL-CEC, in people with and without metabolic disease.

1.11 Thesis Hypothesis:

The primary hypothesis of this thesis is that people with metabolic diseases including obesity, T1DM, and T2DM, exhibit increased CVD risk due to profound HDL dysfunction, that is not captured in the measurement of static HDL-C levels.

A secondary hypothesis of this thesis was that HDL-associated proteins would serve as novel surrogate biomarkers of HDL dysfunction which may be impacted by sex and/or metabolic disease status.

1.11.1 Thesis Aims:

1. To investigate the relationship between HDL-associated proteins and HDL-CEC, and develop novel biomarker panels for total CEC, ABCA1-dependent CEC and ABCA1-independent CEC (Chapter 3).
2. To investigate the impact of metabolic disease on HDL sub-fraction proteomic composition, efflux capacity and particle size to gain greater understanding of residual CVD risk present in these individual populations (Chapter 4).
3. To investigate sex-specific differences in HDL protein composition and function in people with and without metabolic disease (Chapter 5).

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Chapter 2
Methods

2.1 Experimental Samples

Participants with T1DM or T2DM or obesity were contacted either by phone or at the time of their scheduled clinic visit. Inclusion criteria for T1DM were as follows: age 20–65 years; BMI ≥18.5 kg/m^2; duration of diabetes of at least 1 year and HbA1c ≤13%. Inclusion criteria for T2DM were as follows: age 20–65 years; BMI ≥18.5 kg/m^2; duration of diabetes of at least 1 year and HbA1c ≤13%. Inclusion criteria for people with obesity were as follows: age 20–65 years; BMI ≥30 kg/m^2; and HbA1c ≤6%. Inclusion criteria for people without obesity were as follows: non-diabetic, age 20–65 years; BMI ≤29.9 kg/m^2; and HbA1c ≤6%. Exclusion criteria were as follows: pregnant or lactating; and recent illness or any chronic illness likely to influence results. The Diabetes Day Centre in Tallaght University Hospital (TUH), Dublin, recruited all people with diabetes. Control samples were obtained from TUH and the obesity clinic at St. Vincent's University Hospital (SVUH), Dublin.

2.1.1 Ethical Approval

All study subjects gave their written signed consent, which was approved by the Research Ethics Committee of TUH and SVUH.

2.2 Study protocol and anthropometric data

All participants were studied after a 12 hour (h) fast, having avoided excessive exercise and alcohol for the previous 24 h. Height was measured with a Harpenden stadiometer. Weight was measured in a hospital gown. Completed in TUH and SVUH.

2.3 Laboratory methods

Plasma levels of total cholesterol, triglycerides (TG) and HDL-C were measured by an enzymatic calorimetric method on the Roche P Module (Roche, Mannheim, Germany). LDL-C was calculated using the Friedewald equation. Additional samples were centrifuged at 3000 rpm for 15 min at 4°C, and serum were stored at −80°C. Completed in TUH and SVUH.

2.4 HDL isolation

2.4.1 Fast Protein Liquid Chromatography (FPLC)

100 µl serum was mixed with 100 µl phosphate-buffered saline (PBS), containing 1 mM ethylenediaminetetraacetic acid (EDTA), and centrifuged at 8000 rpm for 3 min. Supernatant was transferred to a fresh tube on ice. 100 µl of sample was injected into the injection valve of the ÄKTA FPLC system (GE Healthcare). The sample processed at 0.5 ml/min through two Superose 6 Increase 10/300 GL columns (GE Healthcare, Sweden) placed sequentially, and

was eluted into microcentrifuge tubes as 44 separate fractions using a Frac 920 collector (GE Healthcare). Cholesterol content of each freshly eluted fraction was measured by commercial assay (LabAssay™ Cholesterol; Fujifilm) as per manufacturer's instructions. Fractions were stored at -80°C for future analysis.

2.4.2 Polyethylene glycol (PEG) precipitation [1]

PEG solution was made to a final concentration of 20% (w/v) PEG (Sigma-Aldrich, Ireland) in 200 mM glycine buffer and adjusted to pH 7.4. 20 µl of PEG solution was added to 50 µl serum (2:5 ratio) and incubated for 20 min at room temperature. Samples were centrifuged at 10,000 rpm for 30 minutes (min) at 4°C resulting in the precipitation of apoB-containing lipoproteins. The apoB-depleted supernatant was transferred to fresh microcentrifuge tubes and stored at -80°C for HDL function analysis.

2.5 HDL function assays

2.5.1 J774.2 macrophages

J774.2 murine macrophages, derived from BALB/c mice (European Collection of Authenticated Cell Cultures), from passages 5-25, were used for all cholesterol efflux capacity (CEC) assays studies. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin-streptomycin (Sigma-Aldrich, Ireland) and 10% foetal calf serum (FCS) (Sigma-Aldrich, Ireland) until 80% confluent, at which point they were passaged or used for experiments.

2.5.2 CEC assays

J774.2 murine macrophages, derived from BALB/c mice (European Collection of Authenticated Cell Cultures), were seeded (7×10^5 cells/ml) onto 96-well plates for 24 h and cultured at 37°C. Cells were labelled with ^{3}H-cholesterol (1 µCi/ml) (Perkin-Elmer Analytical Sciences, Ireland) in Roswell Park Memorial Institute (RPMI) media containing 1% FBS for 24 h before being equilibrated for 18 h in DMEM containing 0.2% bovine serum albumin (BSA), with or without cyclic adenosine monophosphate (cAMP) (0.3 mmol/l). J774 macrophages do not express ABCA1 basally, and stimulation with cAMP specifically drives ABCA1 protein expression. *Ex vivo* efflux from labelled macrophages to cholesterol acceptors, 2% pooled human serum (PHS) or 2.8% apolipoprotein (Apo) B-depleted serum following PEG precipitation, were incubated with the cells in minimum essential media (MEM) for 4 h. ^{3}H-cholesterol remaining in cells was calculated after overnight lipid extraction using isopropanol. ^{3}H-cholesterol label counts in media, and isopropanol were measured by liquid scintillation counting; counts were collated to determine the total levels of ^{3}H-cholesterol within the cells at

baseline. Control cells were incubated in MEM alone to establish non-specific efflux, and this count was subtracted from all other counts. Total efflux was determined from cells treated +cAMP. The difference in efflux to HDL from cells stimulated in the presence and absence of cAMP was taken to represent ABCA1-dependent CEC, while ABCA1-independent CEC was calculated from cAMP naive cells.

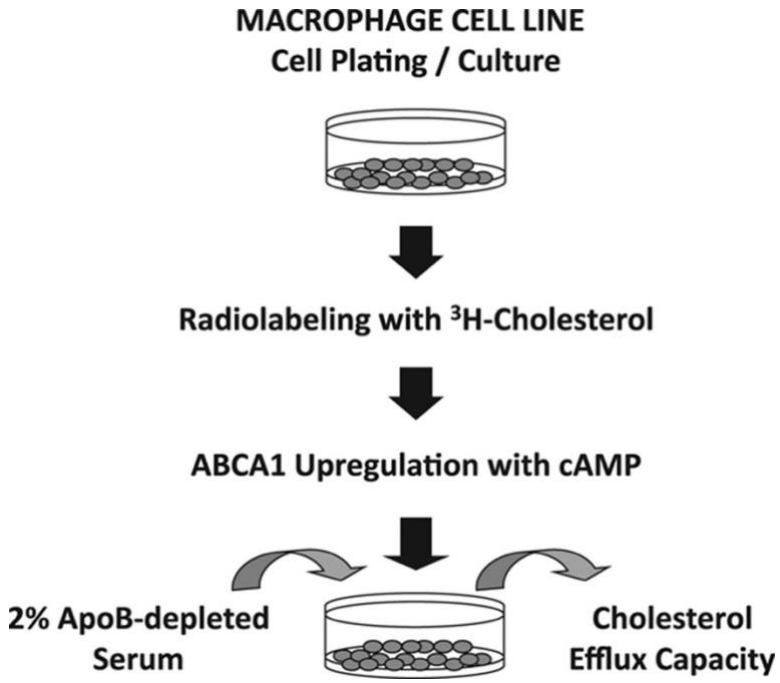

Figure 2.1 *Cholesterol efflux capacity assay [2].*
Schematic diagram of the generic assay of cholesterol efflux capacity (CEC). Macrophages are plated, followed by incubation with radiolabelled cholesterol. cAMP is used to upregulate macrophage expression of the ABCA1 receptor. An individual's serum depleted of ApoB particles, using PEG precipitation, is applied to the cells. After 4 hours, media are harvested. Isopropanol is added to cells to extract lipid. ^{3}H-cholesterol label counts in media, and isopropanol are measured by liquid scintillation counting. Cholesterol efflux capacity is expressed as a percentage. Image taken from Khera *et al.,* [2].

2.5.3 Paraoxonase (PON) 1 activity assay [3]

Paraoxonase buffer (112.43 µl,) which comprised of 2 mM $CaCl_2$, 10 mM Tris-HCl and 1 M NaCl, adjusted to pH 8, was added to 5 µl serum in a 96 well plate. Paraoxon substrate (82.57 µl), at a 1:1000 ratio of paraoxon ethyl (Sigma-Aldrich): paraoxonase buffer, was added to serum. Negative control and blank control were set up as buffer only, and buffer plus paraoxon substrate respectively. The hydrolysis of paraoxon to 4-nitrophenol was measured, at 405nm at time 0 and then read every 2.5 min until 22.5 min, with the ClarioStar (BMG Labtech) plate reader. Enzyme activity was calculated as one unit (U) of PON1 activity, defined by 1 U of 4-nitrophenol formed per ml per minute (U/ml/min). This method was used as a surrogate measurement of the antioxidant function of HDL.

2.6 Nuclear magnetic resonance (NMR) [4]

Lipoprotein particle concentration and sizes were measured in serum samples using 400 MHZ NMR spectroscopy (LipoScience, Raleigh, NC, USA). Each lipoprotein subclass emits a distinctive lipid methyl group NMR signal and the amplitudes of the measured signals are directly proportional to the number of subclass particles, which in turn provides the estimated mol/l concentration for the three HDL particle subclasses (large 8.9 – 13 nm, medium 8.3 – 8.8 nm, small 7.3 – 8.2 nm) [5]. Weighted-average HDL particle sizes in nanometres were calculated from the subclass levels, and the diameters were assigned to each subclass.

2.7 Proteomics sample preparation

2.7.1 Protein isolation

HDL samples were isolated from human serum by FPLC, the HDL containing fractions were then pooled and purified using 30 µl lipid removal agent (LRA) (100mg/ml in 50mM ammonium bicarbonate, Sigma-Aldrich, Ireland). LRA binds phospholipids, which are predominantly associated with HDL in FPLC fractions, and thereby provides a method to further enrich FPLC fractions for HDL [6].

Samples were mixed gently for 30 min at room temperature in the thermomixer. Samples were centrifuged at 5000 rpm for 2 min, and supernatant was discarded. The LRA pellet was resuspended in 25 µl 50 mM ammonium bicarbonate containing 2 M Urea, and samples stored at -80°C.

2.7.2 In-solution digestion

100 mM dithiothreitol (DTT) (1:20 ratio) was added to samples at 60°C for 30 min to reduce the disulphide bonds of the serum protein samples. 200 mM iodoacetamide (IAA) (1:20 ratio) was then added to alkylate the samples. Samples were incubated in the dark at room temperature for 30 min. Samples were diluted using 50 mM ammonium bicarbonate to bring the final concentration of urea to under 2 M. 1 vial of trypsin singles (1 µg size, proteomic grade T7575, Sigma Aldrich) was solubilised with 2 µl trypsin solubilisation solution and diluted with 48 µl 50 mM ammonium bicarbonate. 25 µl trypsin was added to each sample, at a protein to trypsin ratio of 50:1 (v/v), and digestion was conducted overnight at 37°C. Formic acid (FA) was added to the samples at a final concentration of 1%, which acidified the peptides and stopped trypsin digestion. Samples were centrifuged at 10,000 rpm for 5 min, and the supernatant was transferred into fresh microcentrifuge tubes. This was repeated to ensure no LRA was present in the supernatant.

Tryptic digests were purified with C_{18}ZipTip (Merck Millipore). The ZipTips were equilibrated with 20 µl acetonitrile (ACN), and 20 µl 0.1% (v/v) FA, followed by binding the peptides to the

column. The peptides were washed with 0.1% (v/v) FA, and eluted with 30 µl 70% (v/v) ACN in 30% of 0.1% (v/v) FA. Peptides were dried down using a CentriVap Concentrator set at 45°C for 60 min. Peptides were resuspended in 3% ACN with 0.1% FA and quantified using the Denovix.

2.8 Mass spectrometry sample preparation

2.8.1 Bruker TimsTof Pro Mass spectrometry

Peptides were diluted to 0.04 mg/ml with 0.1% FA before being transferred to MS vials. Peptide fractions were analysed on a Bruker TimsTof Pro mass spectrometer connected to a Bruker nano Elute nano-LC chromatography system. 5 µl peptide sample was loaded onto an Aurora UHPLC column. Peptides were eluted with an increasing acetonitrile gradient over 60 min at a flow rate of 250 nl/min at 45°C. The mass spectrometer was operated in positive ion mode. The instrument was operating in trapped ion mobility spectrometry (TIMS) mode when data was acquired. Trapped ions were selected for MS/MS using parallel accumulation serial fragmentation (PASEF).

2.8.2 Quadripole Orbitrap mass spectrometry

Peptides were analysed on a quadripole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer fitted with a reversed-phase nano-LC UltiMate 3000 high performance liquid chromatography (HPLC) system (Thermo Scientific). 5 µl peptide sample was injected into C18 reversed phase columns, 10 cm in length, 75 µm inner diameter. Peptides were eluted with a linear gradient from 2 to 30% acetonitrile containing 0.5% acetic acid in 120 min at a flow rate of 250 nl/min. The Orbitrap, operating in data dependent mode, automatically altered between MS and MS2 acquisition. Survey full scan MS spectra (m/z 350-1600) had a resolution of 70,000. MS2 spectra had a resolution of 17,500. The twelve most intense ions were sequentially isolated and fragmented via higher energy C-trap dissociation.

MS was conducted by Dr. Eugène Dillon in the UCD Conway Proteomics Core.

2.9 Identification and data analysis

2.9.1 Peptide and protein identification

Raw data from the mass spectrometer was processed using MaxQuant software [7] incorporating the Andromeda search engine [8]. To identify peptides and proteins, MS/MS spectra were matched to the Uniprot *homo sapiens* database. All searches were performed with tryptic specificity allowing for two missed cleavages. Mass spectra were searched using the default setting of MaxQuant of a false discovery rate of 1% on the peptide and protein level.

Label-free quantification (LFQ) ion intensities for protein profiles were generated using signals of corresponding peptides in different nano-HPLC. MS/MS runs were matched by MaxQuant applying a mass accuracy of at least 20 ppm, with a maximum time window of 1 min [9].

2.9.2 Proteomic data analysis

Perseus statistical software version 1.6.5.0 was used to analyse the LFQ intensities. Protein identifications were filtered to exclude the reverse database and common contaminants. Data were log transformed and t-test comparisons of groups/samples were conducted. Missing values were imputed with values from the normal distribution to generate heatmaps [10]. The data were normalised via z-score.

2.10 Statistical Analysis

Results in tables are presented as mean ± standard deviation (SD) unless otherwise stated. Data in graphs are presented as mean ± SD. Data were tested for normality using the Shapiro–Wilk test, as well as inspected visually using histograms. Statistical significance for categorical variables was calculated using the χ^2 test. For continuous variables of normally distributed data, the paired or unpaired Student's t test was used to assess statistical significance between two groups as appropriate, while for non-normally distributed data the Wilcoxon Signed Rank test or Mann-Whitney U t-test was applied as appropriate. For multiple comparisons on normal data, paired one-way analysis of variance (ANOVA) tests were performed with a Bonferroni post-hoc test. The Kruskal-Wallis test with a Dunn's multiple comparison post-test was performed on non-normal data. Correlations were evaluated using the Pearson correlation (normal data) or Spearman test (non-normal data) as appropriate. Multiple regression analysis was used to examine the relationship between several independent variables on one dependent variable.

IBM SPSS Statistics (Version 24.0. Armonk, NY) and GraphPad Prism (Version 9.3.0 San Diego, CA) software for Mac were used for data analysis. Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, with significance between groups indicated in each chapter. P>0.05 was considered non-significant.

Chapter-specific methods are described in detail within each chapter.

2.11 References

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Chapter 3

The potential of the HDL proteome to predict HDL-cholesterol efflux capacity in people with and without metabolic disease.

Rachel Byrne, Eugene Dillon, Anna Antoniadi, Mohamed Ahmed, Khalid Ahmed, Anne McGowan, Anjuli Gunness, Catherine Mooney, James Gibney, and Fiona McGillicuddy.

Abstract

Introduction: High-density lipoprotein (HDL) cholesterol efflux capacity (CEC) is a key athero-protective function of HDL that is driven by the interaction with cellular cholesterol transporters including ABCA1, ABCG1 and SR-B1. Measuring HDL-CEC within a cell-culture model is a stronger predictor of CVD events than static HDL-cholesterol (HDL-C). Within this study we sought to identify key proteins on HDL that might predict HDL-CEC in people with and without metabolic disease that could serve as surrogate biomarkers of HDL-CEC.

Methods: Individuals with high HDL-CEC (range: 12.54-19.17%) were age-, sex- and BMI-matched to individuals with low HDL-CEC (range: 6.32-11.7%) across a population of people ±obesity and ±T2DM (*n*=60 total). Total, ABCA1-dependent, and ABCA1-independent efflux were determined after 4h incubation of PEG-supernatants with ^{3}H-cholesterol labeled J774 macrophages treated ± cAMP *ex vivo*. HDL protein composition was investigated by discovery proteomics after fast-protein liquid chromatography isolation and lipid removal agent enrichment of HDL-containing fractions. A scoring algorithm was created for each HDL-CEC parameter (total CEC, ABCA1-dependent CEC, and ABCA1-independent CEC) from the proteins that were identified as significantly correlating with each respective efflux parameter, using Z-scores generated from raw LFQ values.

Results: HDL proteomics analysis identified 25 significantly different proteins between low HDL-CEC and high HDL-CEC effluxers, with reduced association of ApoC-III, ApoC-IV, platelet glycoprotein V and vitamin-K-dependent protein C on HDL in low-effluxers vs. high-effluxers. Reductions in HDL-associated platelet glycoprotein V and vitamin-K-dependent protein C were more pronounced in low-effluxers with T2DM relative to other groups. Combining the significantly different proteins into a total efflux score (r=0.670, *P*<0.0001****), ABCA1-dependent efflux score (r=0.676, *P*<0.0001****) or ABCA1-independent efflux score (r=0.626, *P*<0.0001****) significantly enhanced the predictive capacity for their respective efflux parameters. Our Receiver Operating Characteristic **(**ROC) AUC in turn was 0.859, *P*=0.0001*** for total efflux score, 0.862, *P*=0.0001*** for ABCA1-dependent efflux score and 0.781, *P*=0.0002** for ABCA1-independent efflux score, highlighting the ability of our score to accurately differentiate between high vs. low HDL-CEC.

Conclusion: Our findings indicate an important role for the HDL proteome in governing HDL-CEC and have identified important protein biomarkers for specific HDL efflux pathways. Our total efflux score and ABCA1-dependent score have the potential be surrogate biomarkers for HDL-CEC (AUC>0.8), while the ABCA1-independent requires further refinement. That withstanding there is residual cholesterol efflux that is not accounted for by the HDL proteome and is likely attributable to other parameters including particle size and/or lipidomic composition.

Abbreviations

ABCA1	ATP-Binding Cassette Subfamily A Member 1
ABCG1	ATP-Binding Cassette Subfamily G, member 1
ACE-I	Angiotensin-Converting Enzyme Inhibitors
Apo	Apolipoprotein
ApoA-I	Apolipoprotein A-I
ARB	Angiotensin Receptor Blockers
AUC	Area Under the Curve
BSA	Bovine Serum Albumin
BMI	Body Mass Index
CAD	Coronary Artery Disease
cAMP	Cyclic Adenosine Monophosphate
CEC	Cholesterol Efflux Capacity
CON	Control
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DGUC	Density Gradient Ultracentrifugation
DPP4	Dipeptidyl Peptidase-4
FPLC	Fast Protein Liquid Chromatography
FPR	False Positive Rate
GLP-1	Glucagon-like Peptide-1
HbA1c	Haemoglobin A1c
HDL	High-Density Lipoprotein
HDL-C	High-Density Lipoprotein-Cholesterol
HDL-P	High-Density Lipoprotein-Particle
HDL-CEC	High-Density Lipoprotein-Cholesterol Efflux Capacity
Ig	Immunoglobulin
JUPITER	Justification for the Use of Statins in Prevention: An Intervention Trail Evaluating Rosuvastatin
LCAT	Lecithin-Cholesterol Acyltransferase
LDL	Low-Density Lipoprotein
LDL-C	Low-Density Lipoprotein-Cholesterol
LFQ	Label-Free Quantification
LRA	Lipid Removal Agent
MetS	Metabolic Syndrome
MHO	Metabolically Healthy Obese

MS	Mass Spectrometry
MUO	Metabolically Unhealthy Obese
NCEP-ATP III	National Cholesterol Education Program – Adult Treatment Panel III
OR	Odds Ratio
PON1	Paraoxonase 1/ Serum Paraoxonase/Arylesterase 1
PLTP	Phospholipid Transfer Protein
ROC	Receiver Operating Characteristic
RCT	Reverse Cholesterol Transport
SAA1	Serum Amyloid A 1
SR-B1	Scavenger Receptor Class B Type 1
SD	Standard Deviation
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TPR	True Positive Rate
vWF	von Willebrand Factor

3.1 Introduction

High-density lipoproteins (HDL) are a heterogenous complex emulsion of over 150 lipids [1], 251 proteins [2], and 304 microRNAs [3]. HDL particles (HDL-P) have distinct functional roles in lipid transport, oxidation, inflammation and immunity [4]. These functions are thought to contribute to the cardiovascular protective properties of HDL. The assigned function of individual HDL-associated proteins map with the functional properties of these particles, indicative that HDL protein composition plays an important role in governing HDL-P functionality.

HDL-cholesterol (HDL-C) has been shown to be a robust, consistent, and independent predictor of cardiovascular disease (CVD), and is included as a core component of risk prediction in both the European and American Heart Associations [5, 6]. However, it is an unsuccessful indicator of the complexity of HDL-P. A simple measurement of HDL-C may mask subtle changes of the HDL-P that influence its function. Measurement of HDL-cholesterol efflux capacity (HDL-CEC), as opposed to absolute HDL-C levels, is a stronger predictor of CVD risk [7-9], highlighting that HDL quality seems to be more important than its quantity of cholesterol.

The central role of HDL in reverse cholesterol transport (RCT), is widely considered the key mechanism responsible for HDL-mediated cardio-protection. In the RCT pathway, HDL accepts cholesterol from peripheral cells including lipid-laden foam cells within atherosclerotic lesions and delivers acquired lipid to the liver, for subsequent elimination in the bile and faeces [10, 11]. HDL-CEC is driven by the interaction with cellular cholesterol transporters including ATP binding cassette subfamily A, member 1 (ABCA1), ATP-binding cassette subfamily G, member 1 (ABCG1) and scavenger receptor class B member 1 (SR-B1). In macrophages, small dense lipid-poor HDL-P potently promote efflux via the ABCA1 transporter, while larger HDL-P interact with ABCG1 and SR-B1 transporters [12], and thus different sized particles interact with specific efflux pathways.

Measurement of total HDL-CEC combines the measurement of efflux to both the small HDL-P (ABCA1-dependent efflux) and large HDL-P (ABCA1-independent efflux) and is the most used measurement of HDL-CEC found in the literature. Using a combined measure of efflux to both large and small HDL-P (total HDL-CEC) may negate important information pertaining to efflux to large versus small HDL-P respectively. Furthermore, ABCA1-specific efflux is impaired in people with acute coronary syndrome and coronary artery disease (CAD) [13]. Similarly, in people with diabetes ABCA1-specific efflux is significantly impaired, while ABCG1-mediated efflux is unimpeded [14]. These findings highlight the importance of measuring efflux to both

large and small particles, as well as total CEC. Biomarkers of total CEC, ABCA1-dependent CEC and ABCA1-independent CEC, are a major unmet need to replace currently used cell-based assays and this was a key focus of the current study.

Traditionally, HDL-CEC is measured *ex vivo* using a cell-based assay in which cultured macrophages are loaded with radiolabelled ^{3}H-cholesterol or fluorescently tagged cholesterol before being treated with cholesterol acceptors. The most common cholesterol acceptor is apolipoprotein (Apo) B-depleted serum isolated by precipitating Apo B-containing lipoprotein particles using polyethylene glycol (PEG) leaving a HDL-enriched supernatant [7-9, 15-17]. This cell-based model has been used to demonstrate that HDL-CEC is independent of HDL-C, and that it is the concentration of preβ-1 HDL that affects ABCA1-dependent CEC [18]. However, the cell-based measure of HDL-CEC is complex and low-throughput. The assays are not standardized, and there is wide variation in the method conducted in the literature between different laboratory groups e.g. different cell-lines of macrophages used (murine J774s [7-9, 15] or human THP-1 cells [16, 17]), and different ways of assessing cholesterol efflux (fluorescence-labelled cholesterol [9], colorimetric assay [17], radiolabelled cholesterol [7, 8, 15, 16]). Different time points and concentration of reagents were also reported across studies [7-9, 15-17]. These variations make it difficult to cross compare studies. Although deployed for clinical studies, a standardised method or biomarker of HDL-CEC to enable larger studies would be welcome.

One promising approach to identify new HDL metrics that capture the cardioprotective properties of HDL is quantitative MS/MS-based HDL proteomics. The proteins identified on HDL generally align with the assigned functions of the particles, highlighting the potential of the HDL proteome to act as a surrogate biomarker of HDL-CEC. Proteomic changes have been proposed to have significant functional consequences [19]. There are subtle but different HDL proteomic footprints/ profiles between people with CAD versus controls [20], and between people with type 2 diabetes mellitus (T2DM) and controls [21], suggesting that potential alterations could impact on HDL quality and subsequent CVD risk.

Advancements in omic technologies have given greater insight into the complexity of HDL-P and have afforded us a great opportunity to gain a more in depth understanding of the inter-relationship between particle composition and function. Within this study, we sought to identify whether individual, or groups of HDL-proteins, could serve as potential biomarkers of HDL-CEC across a population of high versus low HDL-CEC effluxers. We investigated whether there are separate biomarker footprints for the various HDL-CEC parameters (total, ABCA1-dependent and ABCA1-independent efflux). We demonstrate that there are changes in the

profile of HDL-associated proteins between people with high HDL-CEC and people with low HDL-CEC, which when combined into a scoring algorithm, strongly correlated with total CEC, ABCA1-dependent CEC, and ABCA1-independent CEC.

Study hypothesis:

The overall study hypothesis is that individual or distinct sets of HDL-associated proteins may serve as novel predictors of HDL-CEC.

Objective:

To identify potential proteins on HDL-P that are correlated to changes in efflux capacity in a cross-sectional population (*n*=60).

Aims:

1. To identify changes in the pattern of HDL-associated proteins between high and low HDL-CEC effluxers in a cross-sectional population of people with and without metabolic disease.
2. To investigate whether combining HDL-associated proteins into a scoring algorithm could further strengthen the predictive capacity for HDL-CEC.
3. To identify protein footprints that predict total CEC, ABCA1-dependent CEC, and ABCA1-independent CEC.

3.2 Methods

3.2.1 Study design and participants

This was a cross-sectional study designed to compare HDL proteomic composition in individuals with high HDL-CEC (*n*=30, range: 12.54-19.17%) to those with low HDL-CEC (*n*=30, range: 6.32-11.7%) who were age, sex and BMI matched. People ±obesity and ±T2DM were included in this study to establish if similar trends in HDL-associated proteins were evident in high and low effluxers with metabolic disease. See **Figure 3.1**.

Participants with T2DM, and participants with obesity were contacted either by phone or at the time of their scheduled clinic visit. Inclusion criteria for T2DM were as follows: age 35–60 years; duration of diabetes of at least 1 year; HbA1c ≥6.5%. Inclusion criteria for people with obesity were as follows: age 35–60 years; BMI ≥30 kg/m^2; and HbA1c ≤6%. Exclusion criteria were as follows: pregnant or lactating; and recent illness or any chronic illness likely to influence results. People without obesity and T2DM were comprised of healthy volunteers on no medications, and were recruited from the general population. Inclusion criteria were as follows: no-diabetes, age 35–60 years; and BMI ≤30 kg/m^2. Exclusion criteria were as for participants with obesity and participants with T2DM. The Diabetes Day Centre in Tallaght University Hospital (TUH), Dublin, recruited all participants.

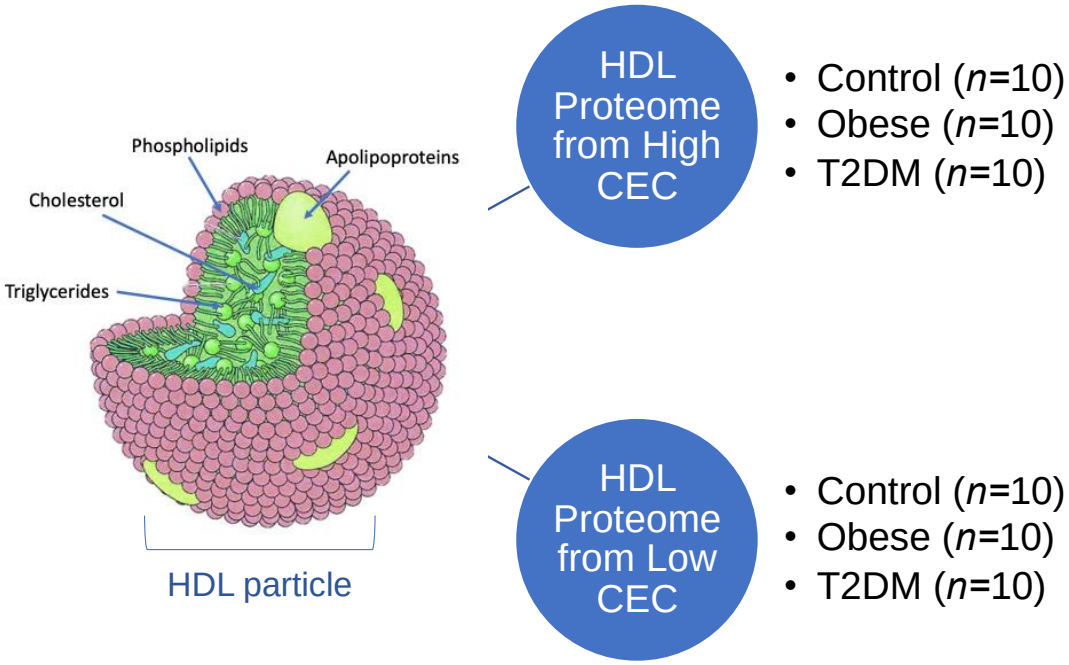


Figure 3.1 *Study design.*

3.2.2 Ethical Approval

All participants gave their written informed consent, which was approved by the Research Ethics Committee of TUH (Dublin, Ireland).

3.2.3 Study protocol and anthropometric data

All participants were studied after a 12 h fast, having avoided excessive exercise and alcohol for the previous 24 h. Height was measured with a Harpenden stadiometer. Weight was measured in a hospital gown. Completed in TUH.

3.2.4 Laboratory methods

Plasma levels of total cholesterol, triglycerides (TG) and HDL-C were measured by an enzymatic calorimetric method on the Roche P Module (Roche, Mannheim, Germany). LDL cholesterol (LDL-C) was calculated using the Friedewald equation. Additional samples were centrifuged at 3000 rpm for 15 min at 4°C, and serum were stored at −80°C. Completed in TUH.

3.2.5 HDL function assays

3.2.5.1 Cholesterol efflux capacity (CEC) [22]

J774.2 murine macrophages, derived from BALB/c mice (European Collection of Authenticated Cell Cultures), were seeded (7×10^5 cells/ml) onto 96-well plates for 24 h, and cultured at 37°C. Cells were subsequently labelled with ^{3}H-cholesterol (1 µCi/ml) for 24 h before being equilibrated for 18 h in DMEM containing 0.2% BSA with or without cAMP (0.3 mmol/l). J774 macrophages do not express ABCA1 basally, and stimulation with cAMP specifically drives ABCA1 protein expression. ApoB-containing lipoproteins were removed from serum by polyethylene glycol (PEG) precipitation, leaving a HDL-enriched supernatant fraction. *Ex vivo* efflux from labelled macrophages to 2.8% HDL-enriched supernatant fraction in minimal essential media (MEM) was measured over 4 h. Control cells were incubated with MEM alone to control for non-specific efflux. ^{3}H-cholesterol levels in the media and remaining cells were calculated by liquid scintillation counting for determination of percentage efflux. Total CEC was determined from cells treated +cAMP. The difference in efflux to HDL from cells stimulated in the presence and absence of cAMP was taken to represent ABCA1-dependent CEC, while ABCA1-independent CEC was calculated from cAMP naive cells.

3.2.5.2 Paraoxonase (PON) 1 activity assay [23]

Paraoxonase buffer (112.43 µl,) which comprised of 2 mM $CaCl_2$, 10 mM Tris-HCl and 1 M NaCl, adjusted to pH 8, was added to 5 µl serum in a 96 well plate. Paraoxon substrate (82.57 µl), at a 1:1000 ratio of paraoxon ethyl (Sigma-Aldrich): paraoxonase buffer, was added to serum. Negative control and blank control were set up as buffer only, and buffer plus paraoxon substrate respectively. The hydrolysis of paraoxon to 4-nitrophenol was measured, at 405nm at time 0 and then read every 2.5 min until 22.5 min, with the ClarioStar (BMG Labtech) plate

reader. Enzyme activity was calculated as one unit (U) of PON1 activity, defined by 1 U of 4-nitrophenol formed per ml per minute (U/ml/min). This method was used as a surrogate measurement of the antioxidant function of HDL.

3.2.6 HDL isolation via fast-protein liquid chromatography (FPLC)

Lipoprotein subpopulations (VLDL, LDL, HDL) were isolated from human serum of all participants. 100 µl of sample, at a serum to PBS containing 1 mM EDTA ratio of 1:1, was injected into the injection valve of the ÄKTA FPLC system (GE Healthcare). The sample processed through two Superose 6 Increase 10/300 GL columns (GE Healthcare, Sweden) placed sequentially, and was eluted into microcentrifuge tubes as 44 separate fractions using a Frac 920 collector (GE Healthcare). Cholesterol content of each freshly eluted fraction was measured by commercial assay (LabAssay™ Cholesterol; Fujifilm) as per manufacturer's instructions. Fractions were stored at -80°C for subsequent analysis.

3.2.7 HDL Proteomics

Sample preparation for proteomics is described in detail in **Chapter 2, Section 2.7.**

3.2.7.1 Protein isolation

HDL containing fractions 36-42 were pooled, and purified using 30 µl lipid removal agent (LRA) (100mg/ml in 50mM ammonium bicarbonate, Sigma-Aldrich, Ireland) [24] to further enrich for phospholipid-containing HDL-P in the fractions. The LRA pellet was resuspended in 25 µl of 50 mM ammonium bicarbonate containing 2 M Urea.

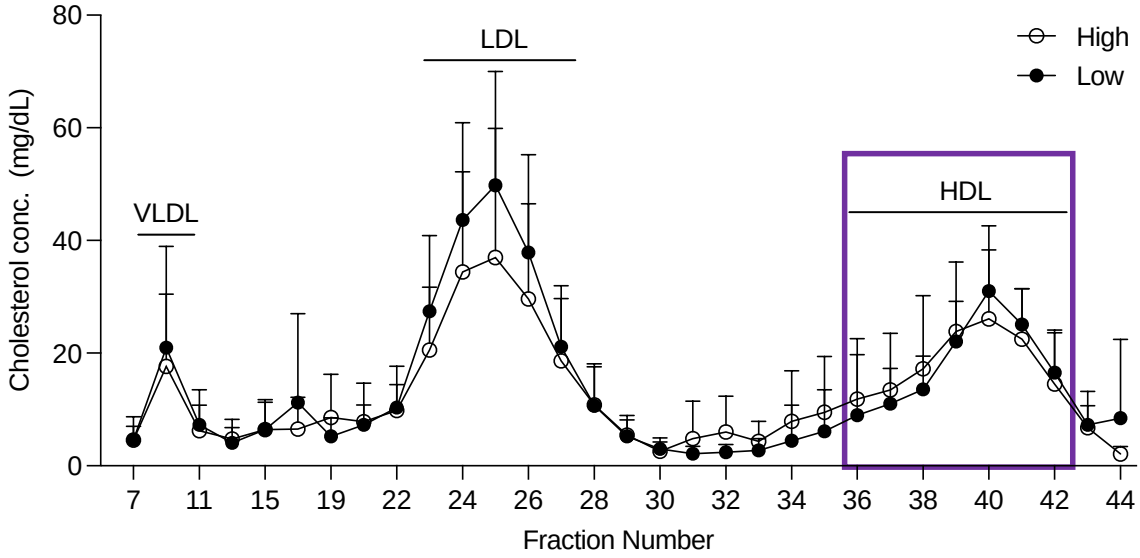


Figure 3.2 *Cholesterol curves of participants with high and low HDL-CEC.*
FPLC was performed on serum samples to separate out the VLDL, LDL and HDL fractions. Total cholesterol mass was measured in each fraction in participants with high (*n*=30) and low (*n*=30) efflux HDL containing fractions 36-42 were pooled, and purified before proteomics preparation. XY graph shows result of cholesterol curves. Data are displayed as mean ± SD.

3.2.7.2 In-solution digestion

Protein samples were in-solution digested with trypsin, (1 vial of trypsin singles at 1 µg size, proteomic grade T7575, Sigma Aldrich, per two samples) at a protein to trypsin ratio of 50:1 (v/v), overnight at 37°C. Peptides were acidified with formic acid (FA), at a final concentration of 1%, to stop trypsin digestion. Tryptic digests were purified using C_{18}ZipTip (Millipore) following the manufactures instructions, and resuspended in 3% acetonitrile (ACN) with 0.1% FA, prior to mass spectrometry (MS) analysis.

3.2.7.3 Mass Spectrometry

Peptide fractions were analyzed on a Bruker TimsTof Pro mass spectrometer connected to a Bruker nano Elute nano-LC chromatography system.

3.2.7.4 Protein identification

Raw data were processed using the MaxQuant version 1.6.10.43, incorporating the Andromeda search engine. To identify peptides and proteins, MS/MS spectra were matched to the Uniprot *homo sapiens* database (2020_06) containing 76,074 entries. In order to generate label free quantitative (LFQ) ion intensities for protein profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant applying a mass accuracy of at least 20 ppm and a maximum time window of 1 min [25].

3.2.7.5 Proteomic data analysis

Perseus statistical software version 1.6.5.0 was used to analyse the LFQ intensities. Data were log transformed, and t-test comparisons of groups conducted. For visualization using heat maps, missing values were imputed with values from a normal distribution, and the z-score was used to normalise the dataset.

3.2.8 Power calculation

Our primary endpoint was to identify protein footprints within the HDL proteome that could differentiate between high and low effluxers across a population with and without metabolic disease. Sample size calculations for HDL proteomics (n=25-30) were calculated based on previous findings of variation in the HDL proteome of people with metabolically healthy obesity (MHO) versus metabolically unhealthy obesity (MUO) (CoV=20.26% and d-value=16.04%). We therefore estimated that n=25 was the minimal number required to see differences across groups.

3.2.9 Statistical Analysis and HDL-CEC score

Results in tables are presented as mean ± SD unless otherwise stated. Data in graphs are presented as mean ± SD. Data were tested for normality using the Shapiro–Wilk test, as well as inspected visually using histograms. Statistical significance for categorical variables was

calculated using the χ^2 test. For continuous variables of normally distributed data, the paired or unpaired Student's t test was used to assess statistical significance between two groups as appropriate, while for non-normally distributed data the Wilcoxon Signed Rank test or Mann-Whitney U t-test was applied as appropriate. Correlations were evaluated using the Pearson correlation (normal data) or Spearman test (non-normal data) as appropriate. Generalized Estimating Equations (GEE) with robust Huber-White (sandwich) standard error estimation was used to examine the joint effect of all predictors while accounting for the non-independence of matched pairs.

To generate a HDL-CEC score for each HDL-CEC parameter from the proteomics database, z-scores were generated from raw LFQ values and the sum of the z-scores of proteins that significantly positively correlated in each HDL-CEC parameter were subtracted from the sum of the z-scores that significantly negatively correlated in each HDL-CEC parameter. These scores were subsequently correlated against the three different HDL-CEC parameters (total, ABCA1-dependent and ABCA1-independent CEC), to establish whether combination of proteins yielded stronger predictive capacity than any given protein alone.

ROC curves were generated to assess the sensitivity and specificity for each of the HDL-CEC scores. For all three of the HDL-CEC scores, the probability threshold/ cut-off point that was used to distinguish between the two groups of high HDL-CEC effluxers vs. low HDL-CEC effluxers was 0.5, which is equivalent to the median value for each of the scores. The sensitivity was calculated by how many cases (people with low HDL-CEC) were correctly identified for each of the HDL-CEC scores, while the specificity measures how many non-cases (people with high HDL-CEC) were correctly identified as non-cases for each of the HDL-CEC scores.

IBM SPSS Statistics (Version 24.0. Armonk, NY), GraphPad Prism (Version 9.3.0 San Diego, CA) and R Foundation for Statistical Computing (www.R-project.org Vienna, Austria) software for Mac were used for data analysis. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC. *P*>0.05 was considered non-significant.

3.3 Results

3.3.1 Baseline characteristics of high vs. low HDL-CEC effluxers

Clinical and biochemical characteristics of 30 participants with high HDL-CEC (range: 12.54-19.17%) and 30 participants with low HDL-CEC (range: 6.32-11.7%) are shown in **Table 3.1**. The medication use in the two groups is in **Table *3.15*** in **Appendix 3A – Medication table**

There were no significant differences in lipid parameters, blood pressure or HbA1c levels between high and low HDL-CEC cohorts. There was a significant difference in the percentage of people who smoked between high and low HDL-CEC cohorts, with a higher percentage of low effluxers who smoke.

There was no difference in overall medication use, with 30% of participants in each group on some form of medication. There were also no differences between the groups with regards to specific medications including angiotensin-converting enzyme inhibitors (ACE-I)/ angiotensin receptor blockers (ARB), gliclazide, dipeptidyl peptidase-4 (DPP4) inhibitors, metformin, glucagon-like peptide-1 (GLP-1) or statins.

Total CEC, ABCA1-dependent and ABCA1-independent efflux were all significant different between the two cohorts and are shown in **Figure 3.3**.

Table 3.1 *Baseline characteristics of high HDL-CEC and low HDL-CEC cohorts.*

Characteristic	High HDL-CEC (*n*=30)	Low HDL-CEC (*n*=30)	95% CI	*P* value
Sex (male/ female) (*n*)	12/18	12/18	N/A	1.0
Age (years)	44.3±7.0	43.2±7.3	−2.6, 4.8	0.55
Body mass index (kg/m^2)	32.3±8	31.7±6.8	−3.2, 4.46	0.743
Smokers (%)	6.7	30	N/A	0.012*
Systolic blood pressure (mm Hg)	126±15.9	126±14.2	−8.55, 9.23	0.939
Diastolic blood pressure (mm Hg)	82±13	80±12	−5.37, 9.33	0.59
Haemoglobin A1c (%)	5.6 (5.4-8.1)	5.6 (5.4-6.7)	−0.58, 1.60	0.348
Total cholesterol (mmol/l)	4.8±1.2	4.7±1.1	−0.6, 0.66	0.932
Triglycerides (mmol/l)	1.25 (0.7-2.6)	1.3 (0.9-1.8)	−1.57, 5.18	0.289
LDL cholesterol (mmol/l)	2.8±1.1	3.0±0.9	−0.79, 0.36	0.452
HDL cholesterol (mmol/l)	1.4±0.4	1.2±0.3	−0.07, 0.33	0.203

Data are displayed as mean±SD, median (IQR) or %
Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

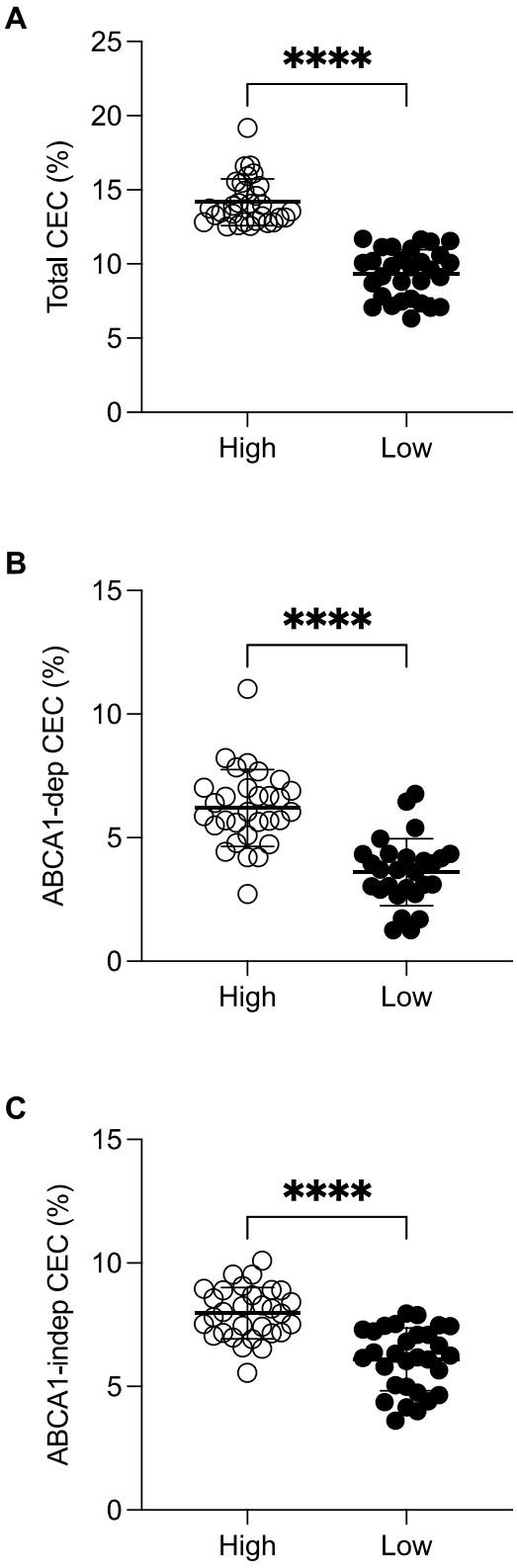


Figure 3.3 *HDL-CEC in participants with high and low HDL-CEC.*
HDL-CEC measurements in participants with high (*n*=30) and low (*n*=30) efflux. J774 macrophages were loaded with ^{3}H-Cholesterol (1µCi/ml) for 24h. Cells were then treated ± cAMP (0.3mM) for 16h and efflux to 2.8% PEG supernatant was monitored over 4h. Dot plots show results for **(A)** total CEC, derived from +cAMP treated cells, **(B)** ABCA1-dependent CEC, calculated as the difference in efflux to PEG-supernatant between cells treated with/ without cAMP**,** and **(C)** ABCA1-independent CEC was calculated from –cAMP treated cells. Data are displayed as mean ± SD. Statistical significance is presented as ****P<0.0001 for high HDL-CEC vs. low HDL-CEC.

3.3.2 Correlations, and univariable and multivariable associations with HDL-CEC parameters

Total CEC was significantly positively correlated to ABCA1-dependent CEC (r=0.856, *P*<0.0001***) and ABCA1-independent CEC (r=0.676, P<0.0001***). ABCA1-dependent CEC, and ABCA1-independent CEC were also significantly correlated to each other but to a lesser extent (r=0.297, *P*=0.025*) (**Table *3.2***). None of the HDL-CEC parameters significantly correlated with any of the lipid parameters (total cholesterol, triglycerides, LDL-C or HDL-C).

Univariable analysis was used to characterise the relationship between the HDL-CEC parameters and age, BMI, sex, smoking and HDL-C separately. Multivariable analysis was then used to assess the influence of the selected variables on HDL-CEC when combined into one model i.e. to assess for confounding effects.

In univariable analysis, total CEC was positively associated with age this effect was lost in the multivariable analysis (**Table *3.3***). Additionally, in univariable analysis, all three HDL-CEC parameters were negatively associated with smoking. This effect remained significant in multivariable analysis for total CEC and ABCA1-dependent CEC, but not for ABCA1-independent CEC. However, in multivariable analysis, ABCA1-independent was negatively associated with BMI, and positively with age and sex (higher in women) (**Table *3.5***).

Table 3.2 *Correlations of metabolic health parameters with HDL-CEC parameters in all participants (n=60).*

Characteristics	r	r^2	*P* value
Total CEC			
Age (years)	0.269	0.072	0.038*
Body mass index (kg/m^2)	0.022	0.0005	0.868
Systolic blood pressure (mm Hg)	−0.0008	0.0001	0.953
Diastolic blood pressure (mm Hg)	0.028	0.001	0.845
Haemoglobin A1c (%)	−0.04	0.002	0.789
Total cholesterol (mmol/l)	−0.026	0.0007	0.853
Triglycerides (mmol/l)	0.099	0.01	0.481
LDL cholesterol (mmol/l)	−0.064	0.004	0.655
HDL cholesterol (mmol/l)	0.042	0.002	0.767
ABCA1-dependent CEC (%CEC/4h)	0.856	0.733	<0.0001***
ABCA1-independent CEC (%CEC/4h)	0.676	0.457	<0.0001***
ABCA1-dependent CEC			
Age (years)	0.209	0.044	0.109
Body mass index (kg/m^2)	0.198	0.039	0.139
Systolic blood pressure (mm Hg)	0.168	0.028	0.244
Diastolic blood pressure (mm Hg)	0.169	0.028	0.241
Haemoglobin A1c (%)	0.113	0.013	0.459
Total cholesterol (mmol/l)	−0.051	0.003	0.729
Triglycerides (mmol/l)	0.143	0.020	0.317
LDL cholesterol (mmol/l)	−0.034	0.001	0.816
HDL cholesterol (mmol/l)	−0.132	0.017	0.362
Total CEC (%CEC/4h)	0.856	0.733	<0.0001***
ABCA1-independent CEC (%CEC/4h)	0.297	0.088	0.025*
ABCA1-independent CEC			
Age (years)	0.217	0.047	0.096
Body mass index (kg/m^2)	−0.130	0.017	0.324
Systolic blood pressure (mm Hg)	−0.211	0.045	0.129
Diastolic blood pressure (mm Hg)	−0.199	0.040	0.153
Haemoglobin A1c (%)	0.019	0.0004	0.896
Total cholesterol (mmol/l)	−0.010	0.0001	0.943
Triglycerides (mmol/l)	−0.019	0.0004	0.0892
LDL cholesterol (mmol/l)	−0.035	0.001	0.809
HDL cholesterol (mmol/l)	0.243	0.059	0.082
Total CEC (%CEC/4h)	0.676	0.457	<0.0001***
ABCA1-dependent CEC (%CEC/4h)	0.297	0.088	0.025*

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3.3 *Univariable and multivariable association of clinical and biochemical variables with total CEC.*

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	0.657*	0.038, 1.276	0.035	−0.028, 0.097
Body mass index (kg/m^2)	0.055	−0.608, 0.719	−0.004	−0.07, 0.062
Sex (1 male; 2 female)	−0.029	−0.073, 0.015	−0.801	−2.248, 0.646
Smoking (1 no; 2 yes)	−0.058***	−0.090, −0.027	−1.406**	−2.371, −0.441
HDL cholesterol (mmol/l)	0.005	−0.030, 0.041	−0.130	−2.038, 1.778

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
β coefficient derived from linear regression and generalised estimating equation

Table 3.4 *Univariable and multivariable association of clinical and biochemical variables with ABCA1-dependent CEC.*

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	0.676	−0.156, 1.509	0.014	−0.049, 0.077
Body mass index (kg/m^2)	0.390	−0.483, 1.262	0.009	−0.069, 0.088
Sex (1 male; 2 female)	−0.042	−0.100, 0.016	−0.715	−1.879, 0.448
Smoking (1 no; 2 yes)	−0.064**	−0.108, −0.021	−1.535*	−2.779, −0.29
HDL cholesterol (mmol/l)	−0.020	−0.068, 0.028	−0.863	−2.845, 1.118

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
β coefficient derived from linear regression and generalised estimating equation

Table 3.5 *Univariable and multivariable association of clinical and biochemical variables with ABCA1-independent CEC.*

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	1.038	−0.191, 2.266	0.107****	0.051, 0.162
Body mass index (kg/m^2)	−0.640	−1.928, 0.648	−0.068*	−0.131, −0.004
Sex (1 male; 2 female)	−0.020	−0.107, 0.068	1.368**	0.532, 2.204
Smoking (1 no; 2 yes)	−0.083*	−148, −0.017	−0.507	−1.299, 0.284
HDL cholesterol (mmol/l)	0.057	−0.008, 0.122	−0.657	−1.974, 0.661

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
β coefficient derived from linear regression and generalised estimating equation

3.3.3 The HDL proteome differs between participants with high vs. low HDL-CEC

We first sought to identify changes in the pattern of HDL-associated proteins between high and low effluxers (*n*=30 per group) across a patient population. 437 valid proteins were detected on HDL after MS. 241 proteins were identified on HDL after filtering for 20 valid proteins (66.7%) in at least one group. 25 significantly different proteins were identified between high HDL-CEC effluxers and low HDL-CEC effluxers. Of these, 10 HDL-associated proteins were significantly increased in low HDL-CEC effluxers relative to high HDL-CEC effluxers including immunoglobulin (Ig) kappa variable 2-30 (IGKV2-30), Ig heavy variable 3-13 (IGHV3-13), and Ig heavy constant alpha 1 (IGHA1), which are involved in the immune response; C4b-binding protein beta chain (C4BPB), and complement C1q subcomponent subunit C (C1QB), which are involved in the complement system; and fibulin-1 (FBLN1), a protein involved in blood coagulation.

By contrast, 15 HDL-associated proteins were significantly reduced in low HDL-CEC effluxers relative to high HDL-CEC effluxers including ApoC-III, a HDL remodelling protein; ApoC-IV, a protein involved in lipid metabolism; beta-ala-his dipeptidase (CNDP1), which is involved in protein metabolism; ApoA-IV, an antioxidant, as well as several proteins involved in the cardiovascular system such as angiotensinogen (AGT), histidine-rich glycoprotein (HRG), hyaluronan-binding protein 2 (HABP2); and others involved in blood coagulation including coagulation factor XII (F12), heparin cofactor 2 (SERPIND1), platelet glycoprotein V (GP5), and vitamin K-dependent protein C (PROC) (**Figure 3.4 A**).

A volcano plot was conducted to display the differences in protein abundance detected between high effluxers and low effluxers using the *P* value >0.05 ($-Log_{10}$=1.3 in plot) as statistical significance and also the false discovery rate (FDR) of 5% ($-Log_{10}$=3 in plot), which adjusts for multiple comparisons in large datasets. IGHV3-13 was the only protein to reach significance when accounting for multiple comparisons (**Figure 3.4 B**) and it's LFQ values are graphed in **Figure 3.7 E.**

HDL-ApoA-I, the most abundant protein on HDL, did not differ between the two groups (**Figure 4.5 A**). Other apolipoproteins including ApoA-II, ApoD and ApoE (**Figure 3.5 B-D**), proteins involved in HDL remodelling including lecithin-cholesterol acyltransferase (LCAT), phospholipid transfer protein (PLTP) and ApoC-II (**Figure 3.6 A-C**) also did not significantly differ between groups. There were also no differences in the HDL antioxidant proteins, serum paraoxonase/arylesterase 1 (PON1) and ApoA-IV (despite significance in the Perseus heatmap), or in the pro-inflammatory proteins serum amyloid A 1 (SAA1) or complement C3, (**Figure 3.7**). The significantly different proteins involved in blood coagulation, blood pressure, angiogenesis and cell adhesion are presented in **Figure 3.8**.

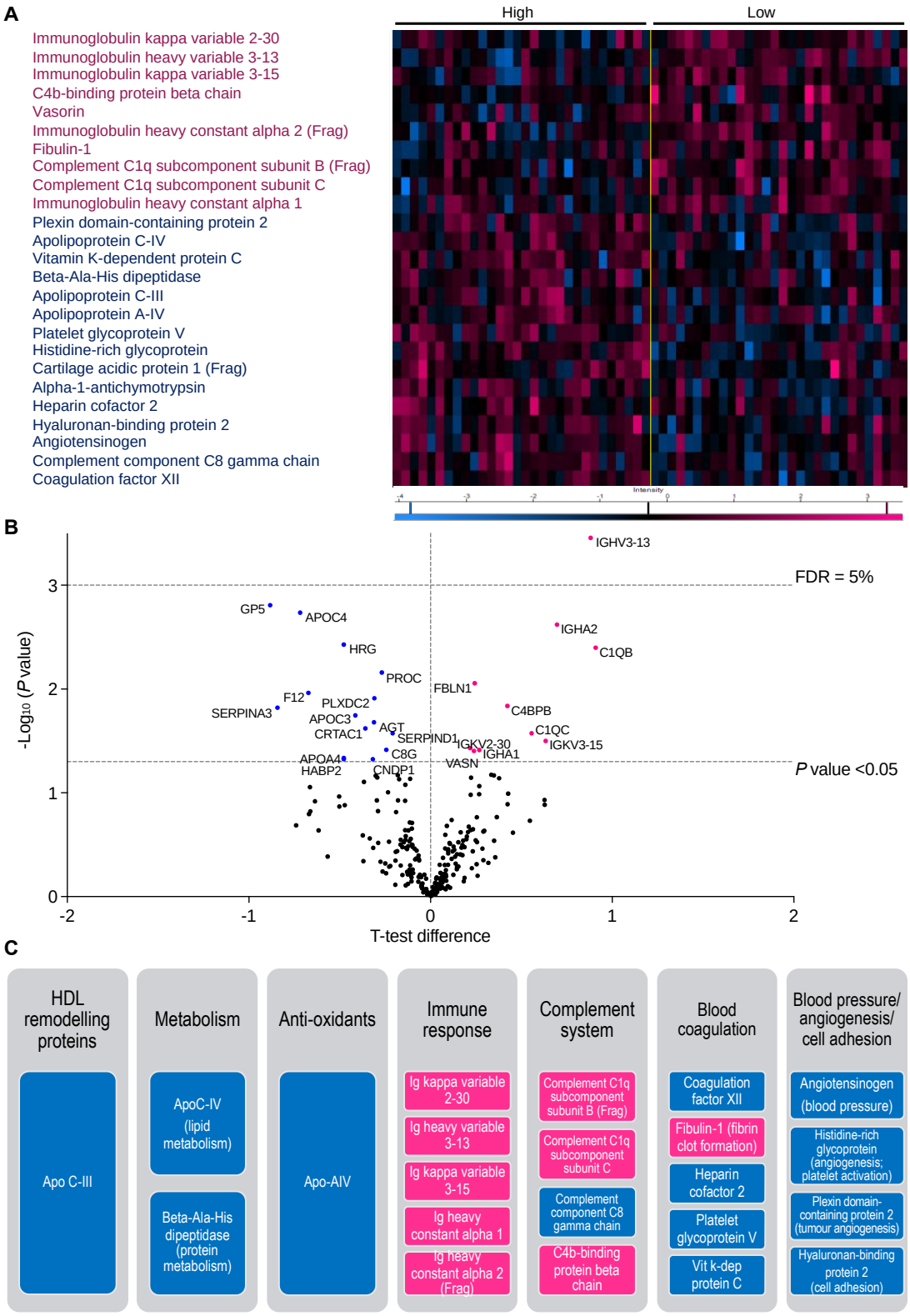


Figure 3.4 *Significantly changed proteins between high HDL-CEC effluxers and low HDL-CEC effluxers and their corresponding functions.*
HDL was isolated via FPLC from people with high HDL-CEC and low HDL-CEC (n=30 per group). Differences in HDL protein levels were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (P<0.05) changed proteins between high and low HDL-CEC effluxers and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between high effluxers and low effluxers. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a P value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. **(C)** Functions of the significantly changed proteins. Pink is up and blue is down in low HDL-CEC vs. high HDL-CEC.

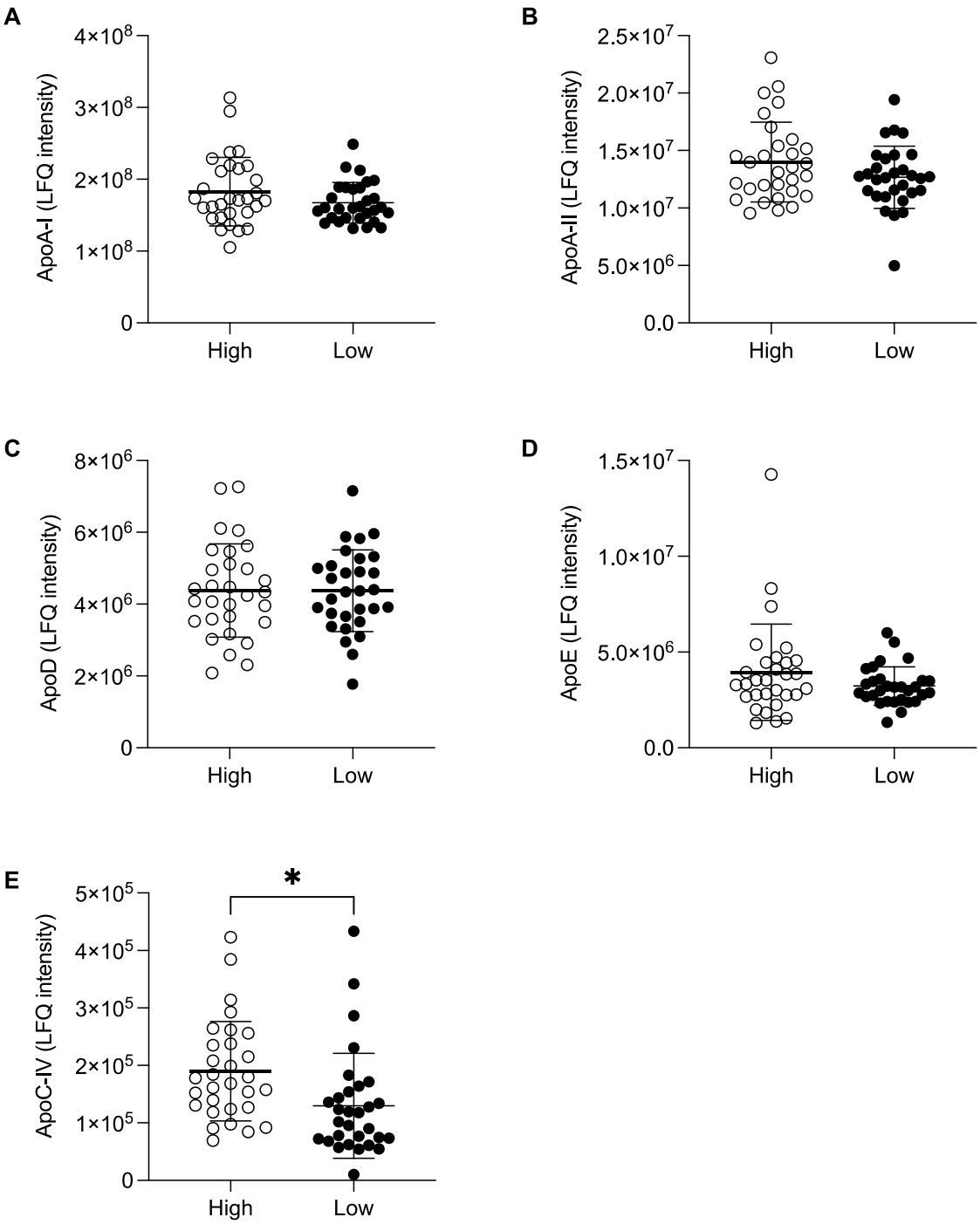


Figure 3.5 *Raw LFQ values of the HDL apolipoproteins in high vs. low HDL-CEC effluxers.* HDL was isolated via FPLC from people with high HDL-CEC and low HDL-CEC (*n*=30 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** ApoA-I, **(B)** ApoA-II, **(C)** ApoD, **(D)** ApoE, and **(E)** ApoC-IV. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

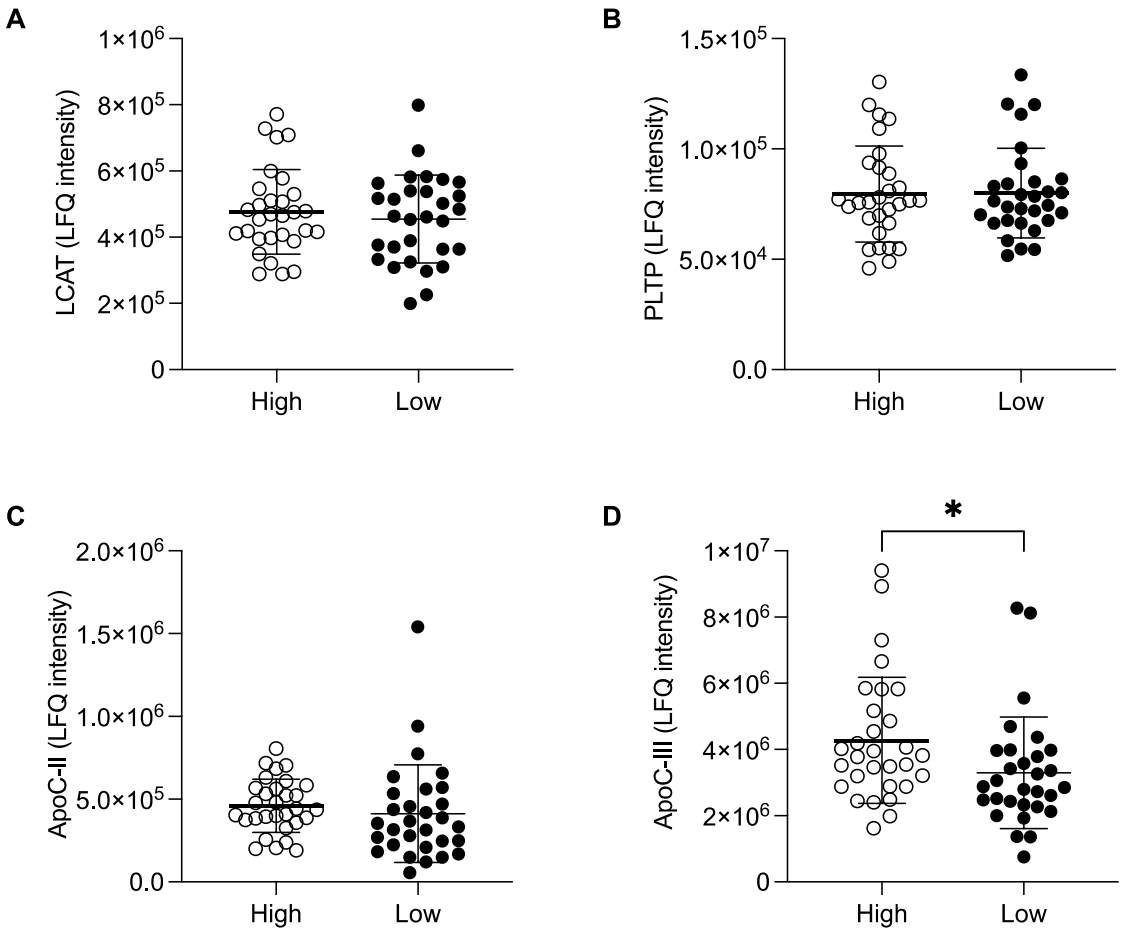


Figure 3.6 *Raw LFQ values of the HDL remodelling proteins in high vs. low HDL-CEC effluxers.*

HDL was isolated via FPLC from people with high HDL-CEC and low HDL-CEC (*n*=30 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** LCAT, **(B)** PLTP, **(C)** ApoC-II, and **(D)** ApoC-III. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

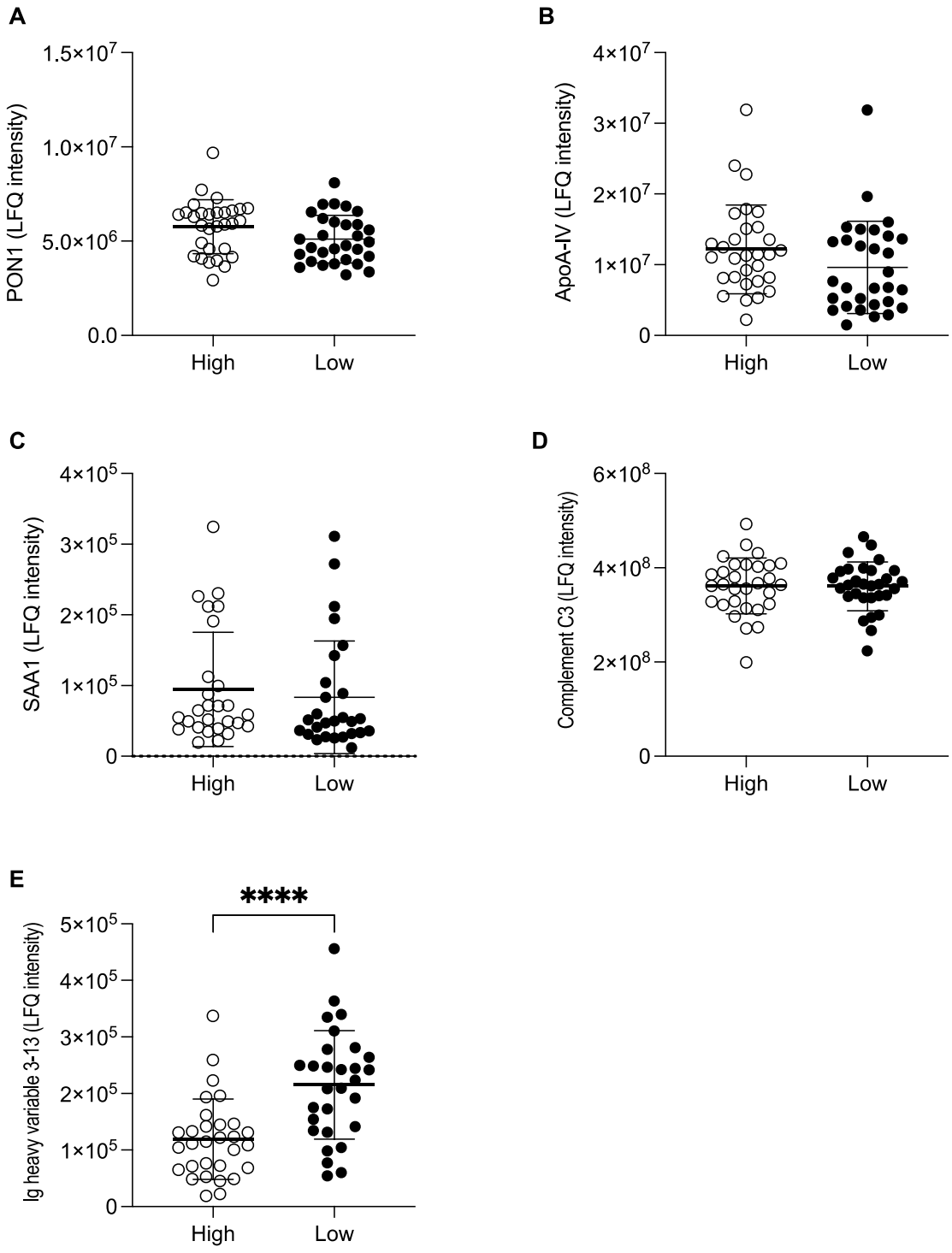


Figure 3.7 *Raw LFQ values of antioxidant, inflammatory and immune proteins in high vs. low HDL-CEC effluxers.*

HDL was isolated via FPLC from people with high HDL-CEC and low HDL-CEC (*n*=30 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** PON1, **(B)** ApoA-IV, **(C)** SAA1, **(D)** Complement C3, and **(E)** Ig heavy variable 3-13. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

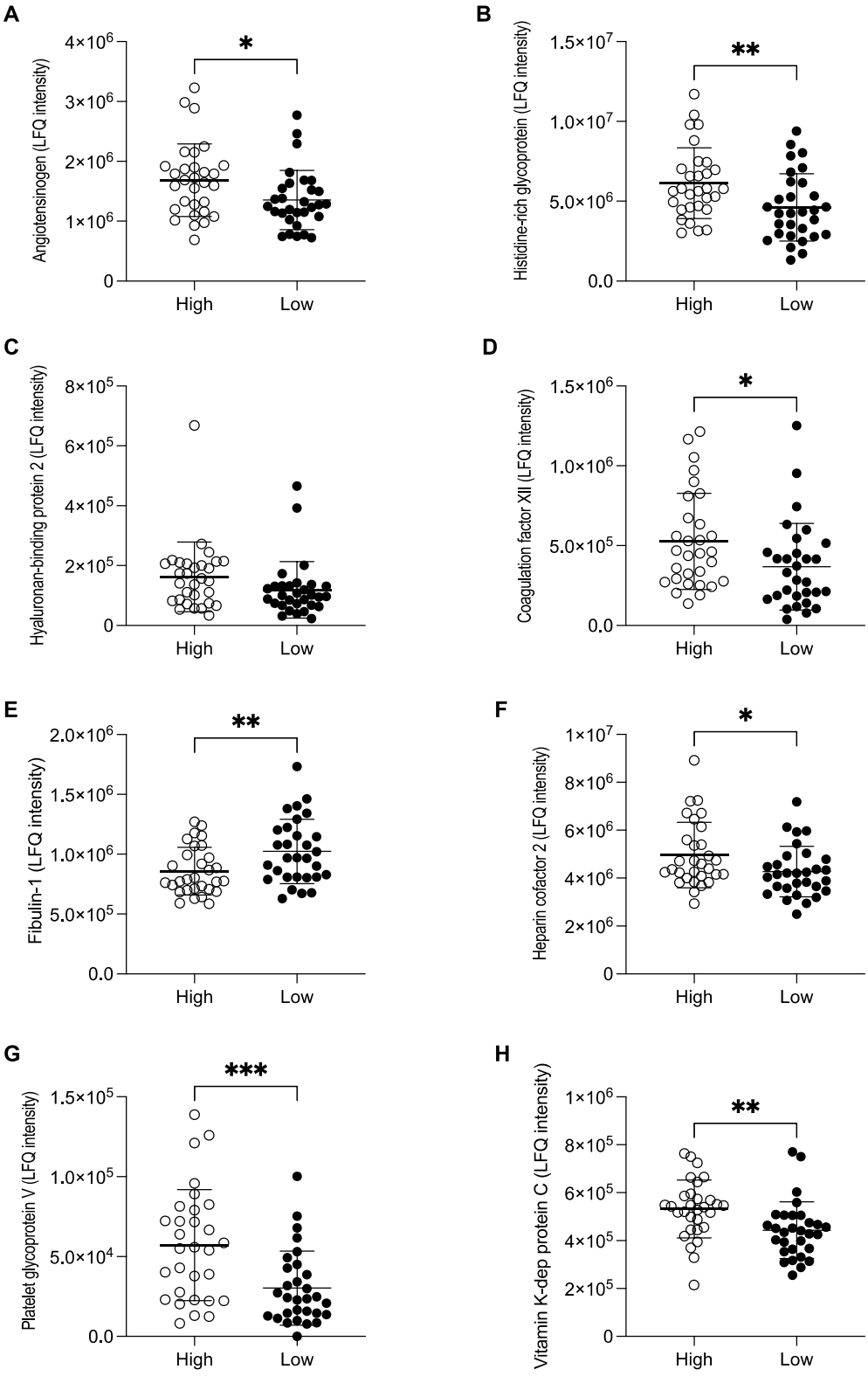


Figure 3.8 *Raw LFQ values of blood coagulation, blood pressure, angiogenesis and cell adhesion proteins in high vs. low HDL-CEC effluxers.*
HDL was isolated via FPLC from people with high HDL-CEC and low HDL-CEC (*n*=30 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** Angiotensinogen, **(B)** Histidine-rich glycoprotein, **(C)** Hyaluronan-binding protein 2, **(D)** Coagulation factor XII, **(E)** Fibulin-1, **(F)** Heparin cofactor 2, **(G)** Platelet glycoprotein V, and **(H)** Vitamin K-dep protein C. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

3.3.4 Baseline characteristics of high vs. low HDL-CEC effluxers subdivided by metabolic disease

The patient population was subsequently divided into those with no obesity or T2DM (CON) (*n*=20), with obesity (OB) (no T2DM) (*n*=20), and with T2DM (*n*=20), to compare the HDL proteome between high HDL-CEC and low HDL-CEC effluxers with and without metabolic disease. It is important to note that the high- and low- HDL-CEC effluxer groups were pair-matched for people with obesity and/or T2DM in this study. We aimed to establish if there were similar changes in the pattern of HDL-associated proteins between high and low effluxers with and without metabolic disease.

Clinical and biochemical characteristics of the 10 participants with high HDL-CEC and 10 participants with low HDL-CEC across all three groups are shown in **Table *3.6*.** The criteria for assessing the metabolic syndrome (MetS) are shown in **Table 3.16** in **Appendix 3B – NCEP-ATP III criteria.** Five out of the 10 participants with obesity in the high efflux group were classified as having metabolically unhealthy obesity (MUO), while the remaining five were classified as having metabolically healthy obesity (MHO). Four out of the 10 participants with obesity in the low efflux group were classified as MUO, while the remaining 6 were classified as MHO.

There were no significant differences in lipid parameters, blood pressure or HbA1c levels between high and low HDL-CEC effluxers across groups. In contrast to the undivided cohort, there was no significant difference in the percentage of people who smoked between high and low HDL-CEC across groups. Total CEC, ABCA1-dependent and ABCA1-independent efflux were all significantly different between the high vs. low effluxers across the three groups and are shown in **Figure 3.9**.

Table 3.6 *Baseline characteristics of high HDL-CEC and low HDL-CEC cohorts split by group.*

Characteristics	High HDL-CEC(*n*=10)	Low HDL-CEC(*n*=10)	95% CI	*P* value
Control				
Sex (male/ female) (*n*)	4/6	4/6	N/A	1.0
Age (years)	44.7±7.2	43.1±7.4	−8.4, 5.2	0.63
Body mass index (kg/m^2)	24.7±1.6	25.3±2.1	−1.2, 2.2	0.52
Smokers (%)	0	10	N/A	0.41
Systolic blood pressure (mm Hg)	117±13	127±14	−4.53, 24.6	0.15
Diastolic blood pressure (mm Hg)	79±10	79±11	−11.3, 10.9	0.97
Haemoglobin A1c (%)	5.2±0.3	5.2±0.4	−0.47, 0.35	0.77
Total cholesterol (mmol/l)	4.7±0.7	4.7±1.3	−0.99, 1.1	0.92
Triglycerides (mmol/l)	0.93±0.54	1.0±0.91	−0.71, 0.94	0.77
LDL cholesterol (mmol/l)	2.6±0.8	2.7±1.1	−0.81, 1.15	0.72
HDL cholesterol (mmol/l)	1.7±0.4	1.5±0.3	−0.57, 0.22	0.35
Obesity				
Sex (male/ female) (*n*)	4/6	4/6	N/A	1.0
Age (years)	43.6±7.1	42.6±8.2	−7.5, 6.1	0.83
Body mass index (kg/m^2)	36.2±6.8	36.2±5.7	−5.8, 5.9	0.99
Smokers (%)	10	30	N/A	0.58
Systolic blood pressure (mm Hg)	134±13	128±13	−16.8, 9.03	0.53
Diastolic blood pressure (mm Hg)	88±13	81±13	−20.3, 5.85	0.26
Haemoglobin A1c (%)	5.5±0.2	5.5±0.1	−0.2, 0.2	1.0
Total cholesterol (mmol/l)	4.9±0.9	5.3±0.9	−0.48, 1.35	0.33
Triglycerides (mmol/l)	1.8±0.90	1.5±0.52	−1.06, 0.44	0.39
LDL cholesterol (mmol/l)	2.9±1.2	3.5±0.7	−0.35, 1.66	0.18
HDL cholesterol (mmol/l)	1.3±0.3	1.1±0.2	−0.44, 0.05	0.11
T2DM				
Sex (male/ female) (*n*)	4/6	4/6	N/A	1.0
Age (years)	44.5±7.4	43.8±7.1	−7.5, 6.1	0.77
Body mass index (kg/m^2)	36.1±7.8	33.6±6.1	−9.1, 4.1	0.44
Smokers (%)	10	50	N/A	0.14
Systolic blood pressure (mm Hg)	131±18	120±16	−34.1, 10.4	0.26
Diastolic blood pressure (mm Hg)	77±14	79±14	−15.5, 20.3	0.77
Haemoglobin A1c (%)	9.1±2.4	7.3±1.3	−3.7, -0.01	0.05
Total cholesterol (mmol/l)	4.8±1.8	4.2±1.0	−1.96, 0.87	0.43
Triglycerides (mmol/l)	2.0±0.97	1.4±0.63	−1.5, 0.16	0.10
LDL cholesterol (mmol/l)	3.0±1.4	2.7±0.8	−1.54, 0.93	0.60
HDL cholesterol (mmol/l)	1.1±0.3	1.1±0.3	−0.21, 0.36	0.57

Data are displayed as mean ± SD, median (IQR) or %
Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

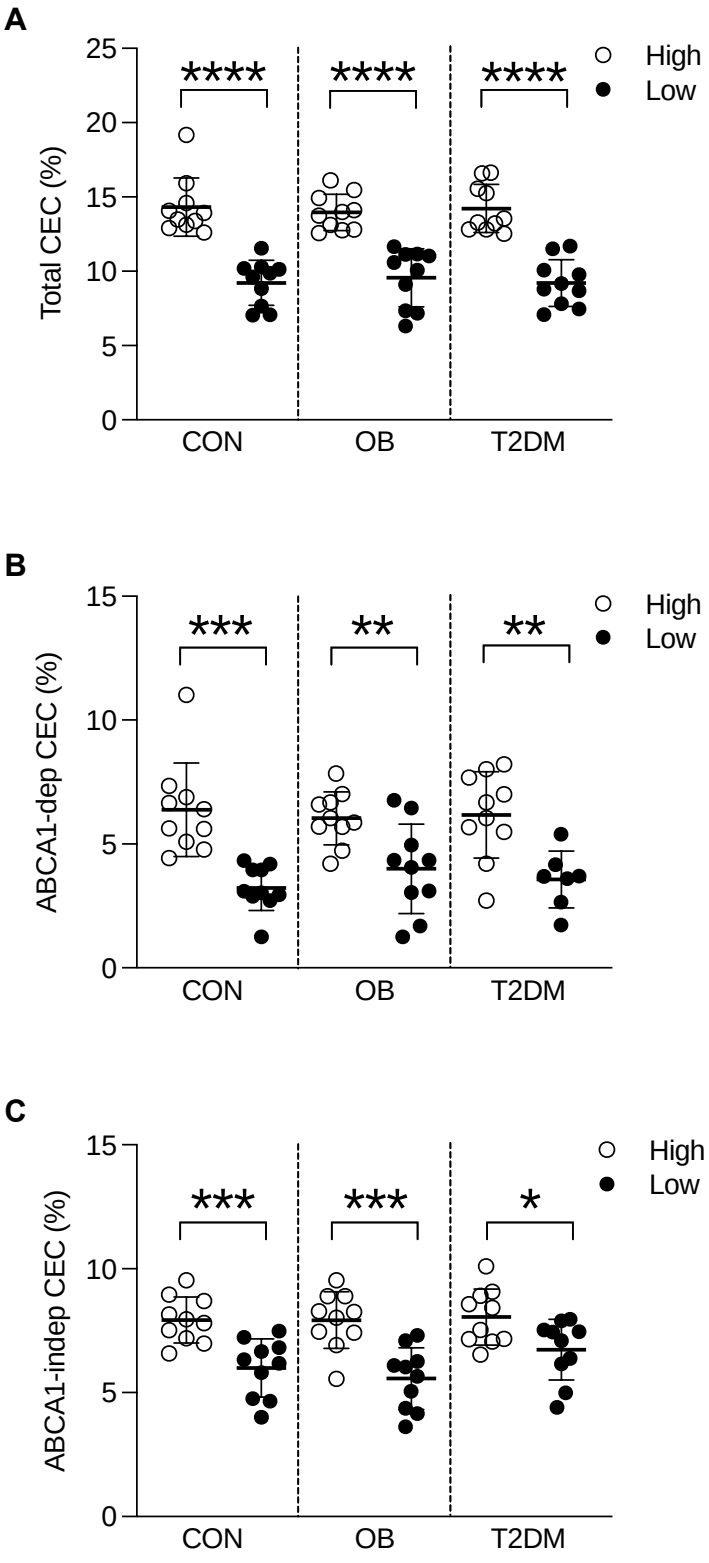


Figure 3.9 *HDL-CEC in participants with high and low HDL-CEC subdivided ±obesity and ±T2DM.*

HDL-CEC measurements in participants with high and low HDL-CEC subdivided into Control (CON), Obesity (OB) and T2DM (n=10 per group). J774 macrophages were loaded with ^{3}H-Cholesterol (1µCi/ml) for 24h. Cells were then treated ± cAMP (0.3mM) for 16h and efflux to 2.8% PEG supernatant was monitored over 4h. Dot plots show results for **(A)** total CEC, derived from +cAMP treated cells, **(B)** ABCA1-dependent CEC, calculated as the difference in efflux to PEG-supernatant between cells treated with/ without cAMP, and **(C)** ABCA1-independent CEC was calculated from –cAMP treated cells Data are displayed as mean ± SD. Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 for high CEC vs. low CEC.

3.3.5 The HDL proteome differs between participants with high vs. low HDL-CEC who have metabolic disease

In individuals without metabolic disease we identified eight significantly different proteins between high HDL-CEC effluxers and low HDL-CEC effluxers (**Figure 3.10**). Of these, three HDL-associated proteins, Ig heavy variable 3-13 (IGHV3-13), Ig heavy constant alpha 2 (frag) (IGHA2) and complement C1q subcomponent subunit B (frag) (C1QB), were significantly increased in low HDL-CEC effluxers compared to high HDL-CEC effluxers, while the remaining five HDL-associated proteins, transforming growth factor-beta-induced protein ig-h3 (TGFBI), Ig lambda variable 2-11 (IGLV2-11), Ig-like domain-containing protein (frag) (IG), insulin-like growth factor-binding protein complex acid labile subunit (IGFALS), and Ig heavy variable 1-58 (IGHV1-58), were significantly decreased.

In people with obesity, we identified eight significantly different proteins between high HDL-CEC effluxers and low HDL-CEC effluxers (**Figure 3.10**). Of these, two HDL-associated proteins, Ig heavy variable 3-13 (IGHV3-13), and Ig kappa variable 3-15 (IGKV3-15) were significantly increased in low HDL-CEC effluxers compared to high HDL-CEC effluxers, while the remaining six HDL-associated proteins, adipocyte plasma membrane-associated protein (frag) (APMAP), PON1, cartilage acidic protein 1 (frag) (CRTAC1), histidine-rich glycoprotein (HRG), ApoC-III, and Ig lambda variable 2-18 (IGLV2-18), were significantly decreased.

In people with T2DM, 11 significantly different proteins were identified between high HDL-CEC effluxers and low HDL-CEC effluxers (**Figure 3.10**). Of these, two HDL-associated proteins Ig heavy constant gamma 3 (IGHG3), and Ig heavy variable 3-13 (IGHV3-13), were significantly increased in low HDL-CEC effluxers compared to high HDL-CEC effluxers while the remaining nine HDL-associated proteins including ApoC-II, ApoC-IV, platelet glycoprotein V (GP5), vitamin K-dependent protein C (PROC), hyaluronan-binding protein 2 (HABP2), complement component C8 gamma chain (C8G), and alpha-2-HS-glycoprotein (AHSG), were significantly decreased. Similar to persons with obesity, HDL-associated PON1 and histidine-rich glycoprotein (HRG) were significantly reduced in low HDL-CEC effluxers relative to high HDL-CEC effluxers in people with T2DM.

It is important to note that the changes in HDL-associated proteins were subtle and when corrections for FDR were made, only one protein (IGLV2-11), reached the significant threshold in controls, and no parameters reached significance in the obese and T2DM group (**Figure 3.11 A-C**).

There were no differences between HDL-ApoA-I, ApoA-II, ApoD and ApoE across the groups (**Figure 3.12 A-D**). There were also no significant differences in key proteins involved in HDL remodelling such as LCAT, and PLTP (**Figure 3.13 A-B**), the HDL antioxidant protein, ApoA-IV, or in the pro-inflammatory proteins SAA1 and complement C3 (**Figure 3.14 B-D**). The

significantly different proteins involved in the blood coagulation, blood pressure, angiogenesis and cell adhesion are presented in **Figure 3.15**. Of interest, reductions in HDL-associated platelet glycoprotein V and vitamin-K dependent protein C were more pronounced in low HDL-CEC effluxers with T2DM than any other group and may represent important biomarkers of residual cardiovascular risk in these individuals that are not accounted for by traditional risk-factors.

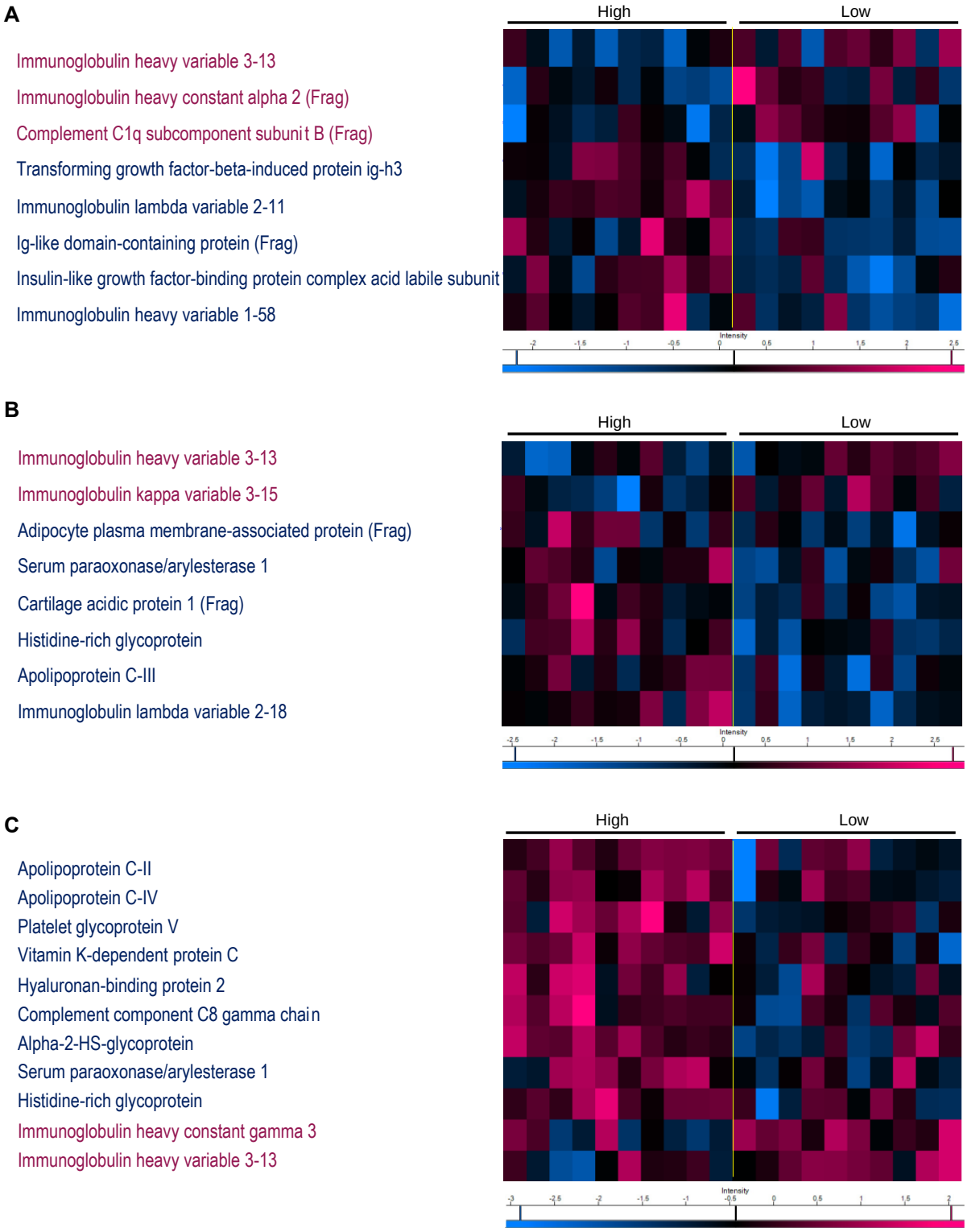


Figure 3.10 *Significantly changed proteins between high HDL-CEC effluxers and low HDL-CEC effluxers subdivided by metabolic disease.*
HDL was isolated via FPLC from participants with high and low HDL-CEC subdivided into Control (CON), Obesity (OB) and T2DM (*n*=10 per group). Differences in HDL protein levels were identified by mass spectrometry. Perseus software was used to determine significantly ($p<0.05$) changed proteins between high and low HDL-CEC effluxers across groups and presented in a heatmap. **(A)** Control, **(B)** Obesity, and **(C)** T2DM.
Pink is up and blue is down.

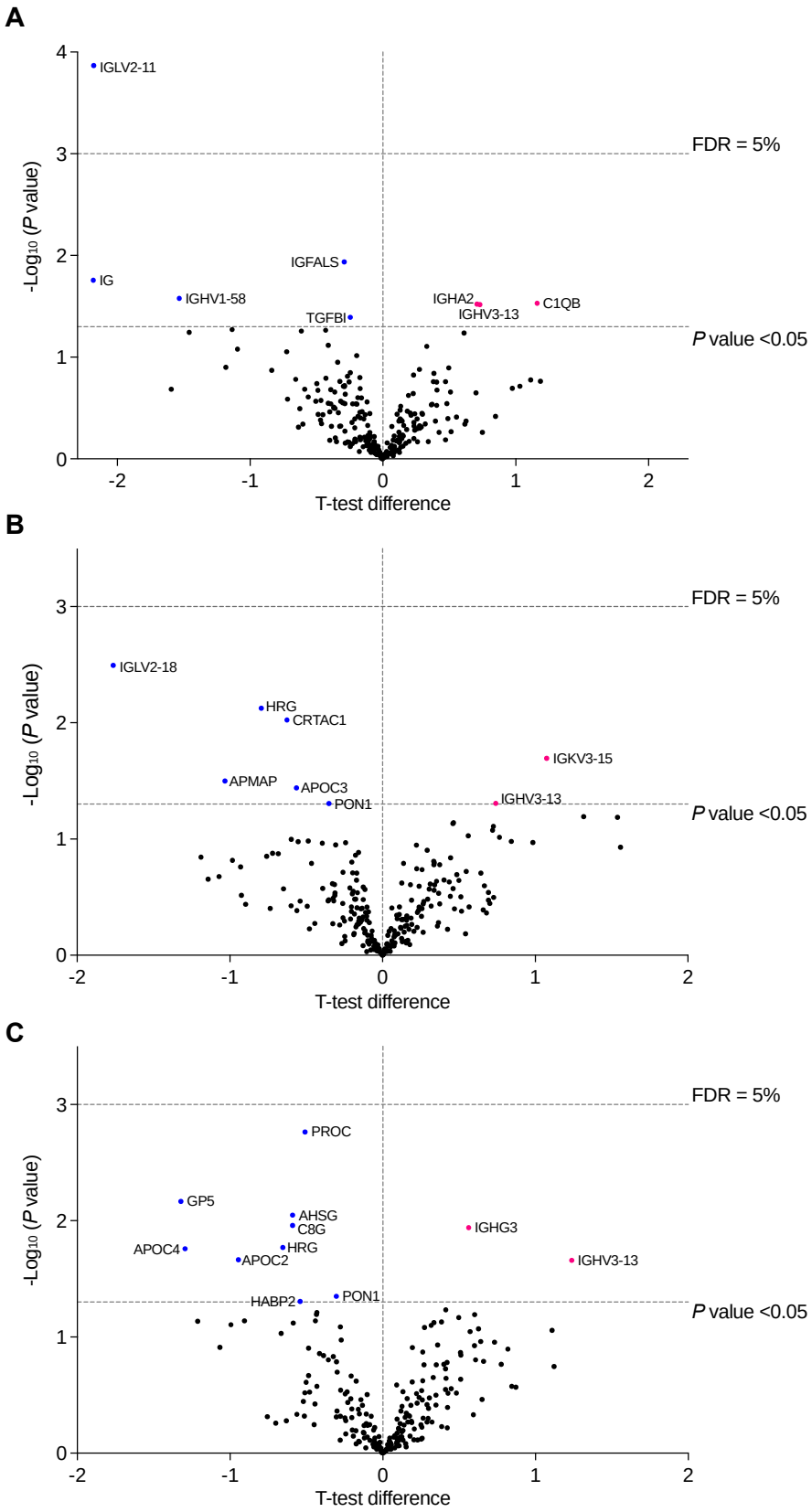


Figure 3.11 *Volcano plot showing differences in protein abundance between high HDL-CEC effluxers and low HDL-CEC effluxers subdivided by metabolic disease.*
Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a P value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. **(A)** Control, **(B)** Obesity, and **(C)** T2DM.
Pink is up and blue is down in low HDL-CEC vs. high HDL-CEC.

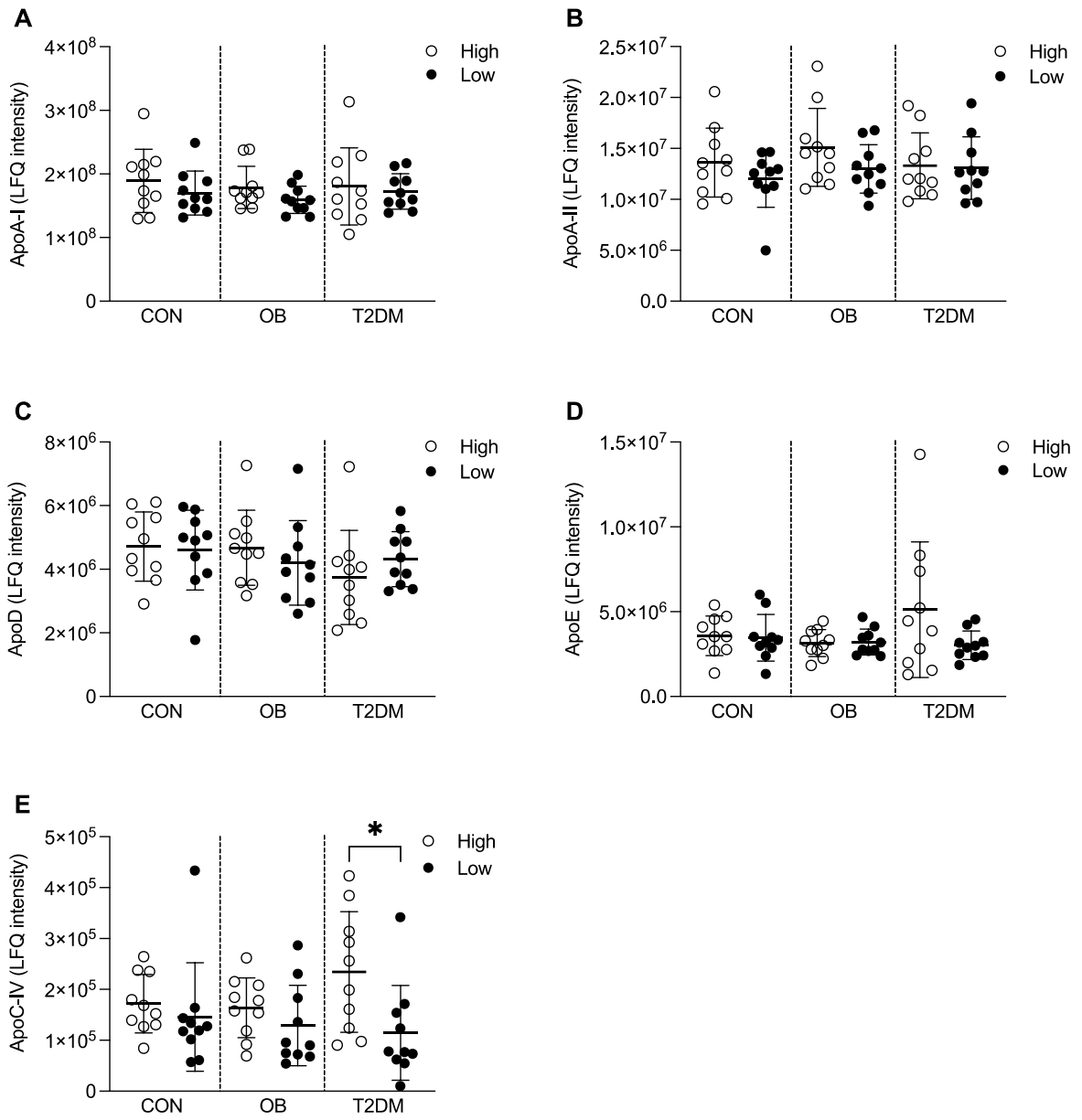


Figure 3.12 *Raw LFQ values of the apolipoproteins between high HDL-CEC effluxers and low HDL-CEC effluxers subdivided by metabolic disease.*

HDL was isolated via FPLC from participants with high and low HDL-CEC subdivided into Control (CON), Obesity (OB) and T2DM (*n*=10 per group). Difference in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** ApoA-I, **(B)** ApoA-II, **(C)** ApoD, **(D)** ApoE, and **(E)** ApoC-IV. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

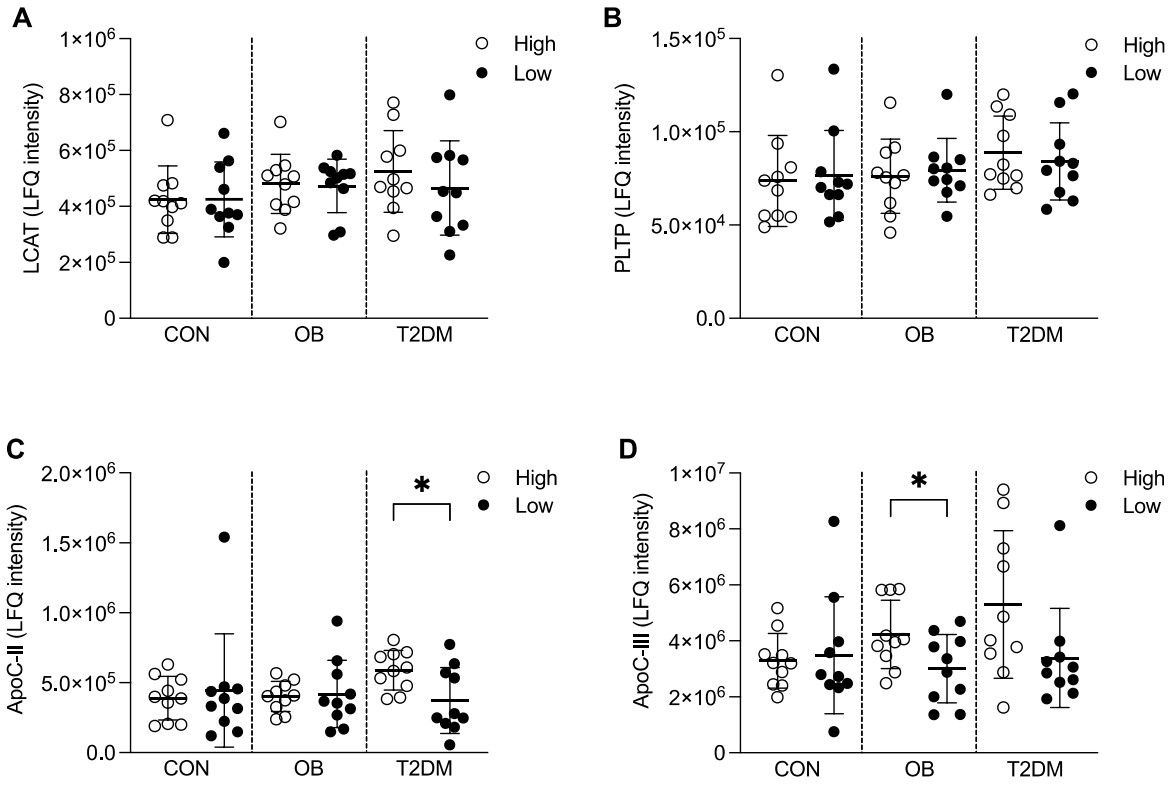


Figure 3.13 *Raw LFQ values of the HDL remodelling proteins between high HDL-CEC effluxers and low HDL-CEC effluxers subdivided by metabolic disease.*
HDL was isolated via FPLC from participants with high and low HDL-CEC subdivided into Control (CON), Obesity (OB) and T2DM (*n*=10 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** LCAT, **(B)** PLTP, **(C)** ApoC-II, and **(D)** ApoC-III. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

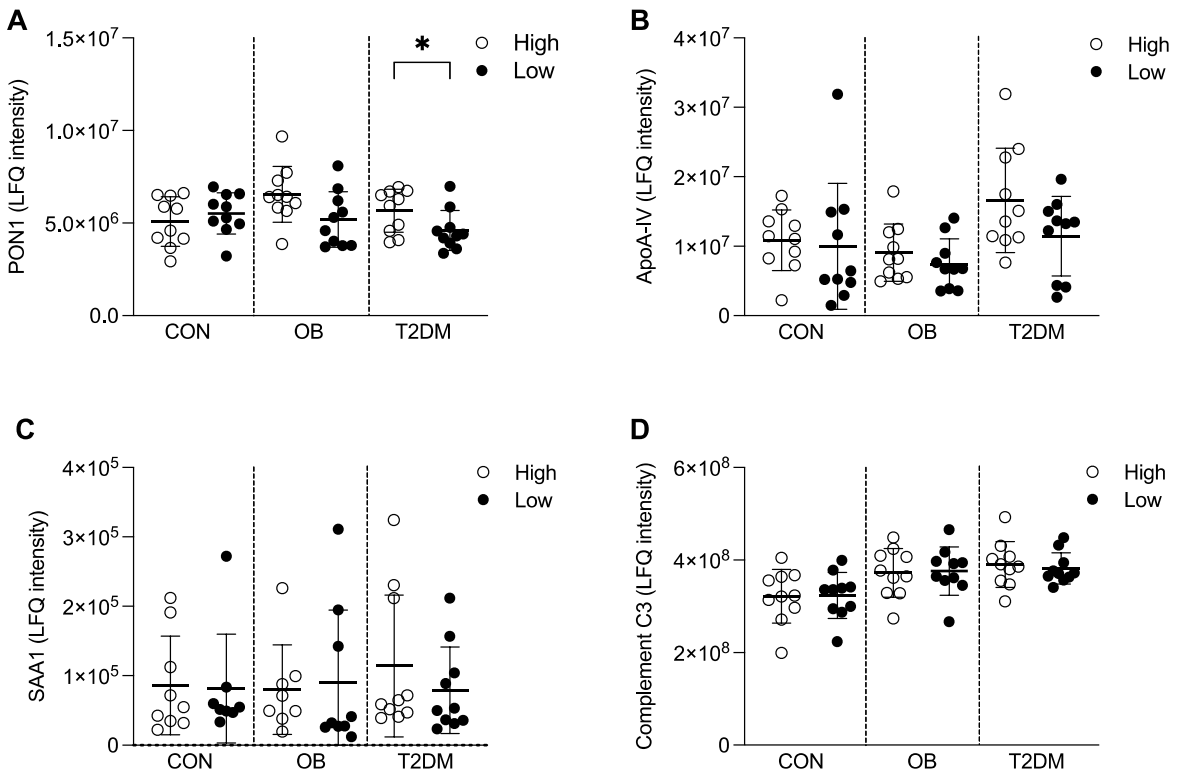


Figure 3.14 *Raw LFQ values of antioxidant, and inflammatory proteins between high HDL-CEC effluxers and low HDL-CEC effluxers subdivided by metabolic disease.*
HDL was isolated via FPLC from participants with high and low HDL-CEC subdivided into Control (CON), Obesity (OB) and T2DM (*n*=10 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** PON1, **(B)** ApoA-IV, **(C)** SAA1, and **(D)** Complement C3. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

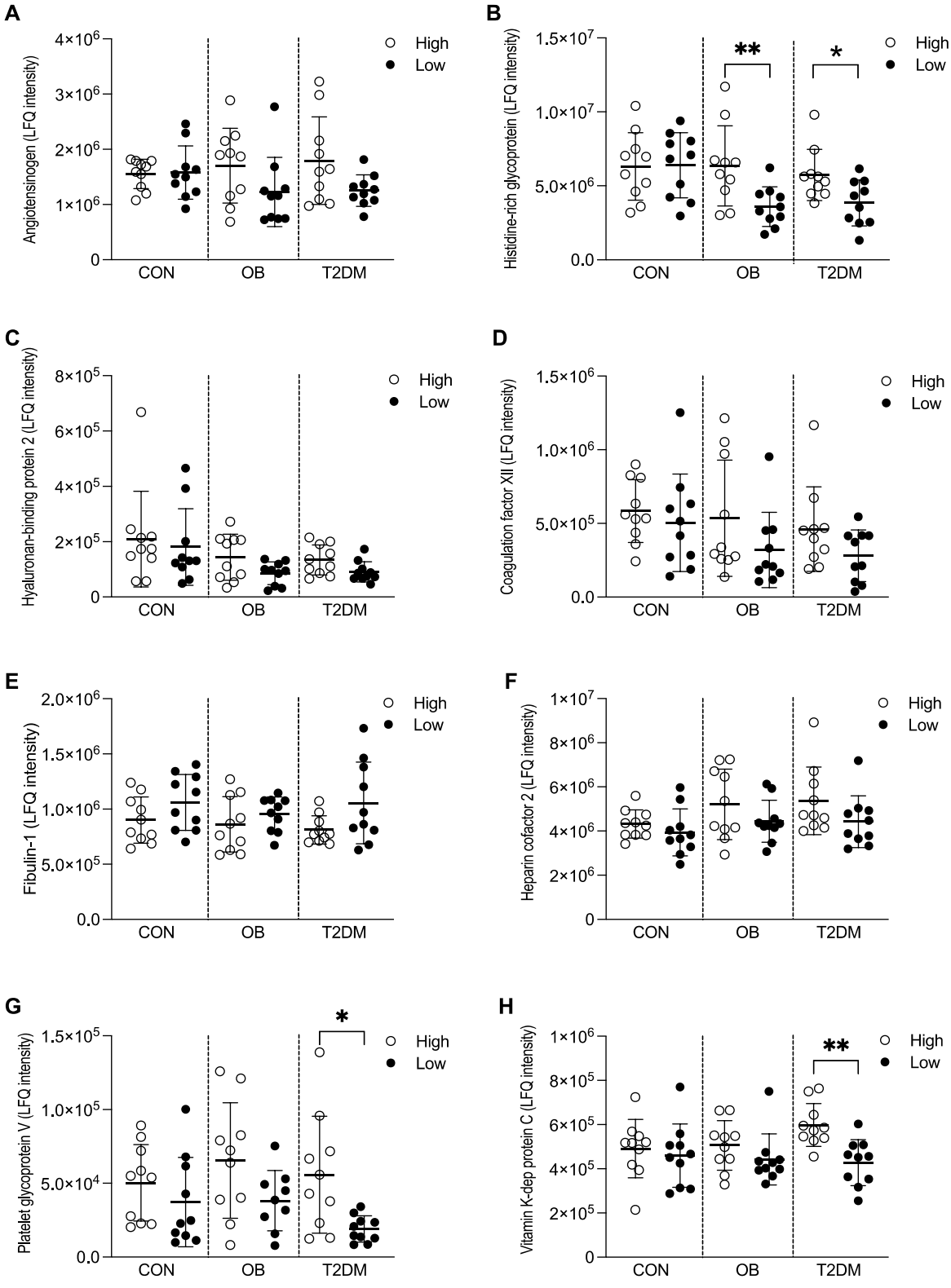


Figure 3.15 *Raw LFQ values of blood coagulation, blood pressure, angiogenesis and cell adhesion proteins between high HDL-CEC effluxers and low HDL-CEC effluxers subdivided by metabolic disease.*

HDL was isolated via FPLC from participants with high and low HDL-CEC subdivided into Control (CON), Obesity (OB) and T2DM (n=10 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** Angiotensinogen, **(B)** Histidine-rich glycoprotein, **(C)** Hyaluronan-binding protein 2, **(D)** Coagulation factor XII, **(E)** Fibulin-1, **(F)** Heparin cofactor 2, **(G)** Platelet glycoprotein V, and **(H)** Vitamin K-dep protein C. Data are displayed as mean ± SD. Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 for high CEC vs. low CEC.

3.3.6 Significant correlations of HDL-associated proteins with HDL-CEC parameters

Correlation analysis was conducted on 225 HDL-associated proteins which were identified on HDL after filtering for seven out of 10 valid proteins (70%) in all groups. **Table 3.7** shows all significantly correlated proteins to HDL-CEC parameters.

Surprisingly, HDL-ApoA-I did not significantly correlate with total CEC (r=0.051, *P*=0.697), ABCA1-dependent CEC (r=−0.159, *P*=0.236) but did with ABCA1-independent CEC (r=0.370, *P*=0.0036**).

HDL-PON1 significantly positively correlated with total CEC (r=0.273, *P*=0.034*) and ABCA1-independent CEC (r=0.332, *P*=0.0096**), but not ABCA1-dependent CEC (r=0.134, *P*=0.321).

By contrast, HDL-ApoB-100 significantly positively correlated with total CEC (r=0.255, *P*=0.0495*) and ABCA1-dependent CEC (r=0.331, *P*=0.012*), but not ABCA1-independent CEC (r=0.065, *P*=0.624).

Complement C1q subcomponent subunit B (Frag) [total CEC (r=0.377, *P*=0.003**), ABCA1-dependent CEC (r=−0.389, *P*=0.003**), and ABCA1-independent CEC (r=−0.269, *P*=0.038*)], Ig heavy variable 3-13 [(total CEC (r=−0.377, *P*=0.003**), ABCA1-dependent CEC (r=−0.309, *P*=0.019*), and ABCA1-independent CEC (r=−0.306, *P*=0.017*)], and vitamin K-dependent protein C [total CEC (r=0.397, *P*=0.002**), ABCA1-dependent CEC (r=0.293, *P*=0.027*), and ABCA1-independent CEC (r=0.306, *P*=0.018*)] were the only proteins that significantly correlated to all three HDL-CEC parameters.

Table 3.7 *Proteins that significantly correlate with efflux parameters.*

HDL-associated protein	Total Efflux	ABCA1 Dependent Efflux	ABCA1 Independent Efflux
Alpha-1-antichymotrypsin	0.318*	0.215	0.213
Angiotensinogen	0.293*	0.153	0.306*
Apolipoprotein A-I	0.051	−0.159	0.370**
Apolipoprotein A-IV	0.129	0.070	0.305*
Apolipoprotein B-100	0.255*	0.331*	0.065
Apolipoprotein C-III	0.298*	0.187	0.260*
Apolipoprotein C-IV	0.325*	0.058	0.399**
Apolipoprotein E	0.287*	0.260	0.226
Beta-Ala-His dipeptidase	0.238	0.131	0.295*
CD5 antigen-like	0.255*	0.286*	0.198
Coagulation factor XII	0.290*	0.206	0.184
Complement C1q subcomponent subunit B (Fragment)	−0.377**	−0.389**	−0.269*
Complement C1q subcomponent subunit C	−0.287*	−0.213	−0.321*
Dopamine beta-hydroxylase	0.049	−0.095	0.263*
EGF-containing fibulin-like extracellular matrix protein 1	−0.347**	−0.129	−0.370**
Endoplasmic reticulum chaperone BIP	0.114	0.060	0.298*
Fibulin-1	−0.308*	−0.324*	−0.142
Glutathione peroxidase	0.231	0.035	0.351**
Hemoglobin subunit alpha	−0.184	−0.270*	−0.024
Hemoglobin subunit beta	−0.169	−0.265*	−0.010
Histidine-rich glycoprotein	0.321*	0.254	0.230
Immunoglobulin heavy constant alpha 1	−0.215	−0.040	−0.364**
Immunoglobulin heavy constant gamma 3	−0.274*	−0.168	−0.408**
Immunoglobulin heavy variable 3-13	−0.377**	−0.309*	−0.306*
Immunoglobulin heavy variable 3-64D	−0.249	−0.299*	−0.205
Immunoglobulin kappa constant	−0.056	−0.035	−0.282*
Immunoglobulin lambda variable 3-10	−0.206	−0.308*	−0.002
Immunoglobulin lambda-like polypeptide 1	0.282*	0.384**	−0.090
Insulin-like growth factor-binding protein complex acid labile subunit	0.278*	0.162	0.128
Kininogen-1	0.202	0.343**	0.203
L-selectin	−0.273*	−0.265*	−0.164
Platelet glycoprotein V	0.326*	0.270*	0.193
Plexin domain-containing protein 2	0.269*	0.106	0.249
Probable non-functional immunoglobulin heavy variable 3-38	−0.084	0.019	−0.276*
Serum paraoxonase/arylesterase 1	0.273*	0.134	0.332**
Sex hormone-binding globulin	−0.102	−0.301*	0.069
Vasorin	−0.261*	−0.332*	−0.024
Vitamin K-dependent protein C	0.397**	0.293*	0.306*

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

3.3.7 HDL-CEC scores – Significantly correlated proteins used to make Z-scores for HDL-CEC parameters

A scoring algorithm was generated based on the significantly correlated HDL-associated proteins to each of the HDL-CEC parameters, creating three different HDL-CEC scores; total efflux score, ABCA1-dependent score and ABCA1-independent score. This was to investigate whether combining HDL-associated proteins into a scoring algorithm could have the capacity to predict HDL-CEC beyond any individual HDL-associated protein.

Table *3.8* shows significantly correlated proteins to total efflux, which were used to generate the total efflux score. **Table *3.9*** shows significantly correlated proteins to ABCA1-dependent efflux which were used to generate the ABCA1-dependent score. **Table *3.10*** shows significantly correlated proteins to ABCA1-independent, which were used to generate the ABCA1-independent score. The lists of contributing proteins to each score are ranked in order of significance.

Each of the three scores for each individual efflux parameter were then plotted according to whether they had high HDL-CEC or low HDL-CEC efflux (**Figure 3.16**). All three scores, the total efflux score, ABCA1-dependent score and ABCA1-independent score were significantly different between high HDL-CEC effluxers and low HDL-CEC effluxers.

Table 3.8 *Significant correlations between HDL-associated proteins and total CEC.*

HDL-associated proteins	r	r^2	*P* value
Positively correlated proteins			
Vitamin K-dependent protein C	0.397	0.158	0.002**
Platelet glycoprotein V	0.326	0.106	0.011*
Apolipoprotein C-IV	0.325	0.106	0.011*
Histidine-rich glycoprotein	0.321	0.103	0.013*
Alpha-1-antichymotrypsin	0.318	0.101	0.013*
Apolipoprotein C-III	0.298	0.089	0.021*
Angiotensinogen	0.293	0.086	0.023*
Coagulation factor XII	0.290	0.084	0.025*
Apolipoprotein E	0.287	0.083	0.026*
Immunoglobulin lambda-like polypeptide 1	0.282	0.079	0.029*
Insulin-like growth factor-binding protein complex acid labile subunit	0.278	0.077	0.032*
Serum paraoxonase/arylesterase 1	0.273	0.075	0.035*
Plexin domain-containing protein 2	0.269	0.072	0.038*
CD5 antigen-like	0.255	0.065	0.0497*
Apolipoprotein B-100	0.255	0.065	0.0495*
Negatively correlated proteins			
Complement C1q subcomponent subunit B (Fragment)	−0.377	0.142	0.003**
Immunoglobulin heavy variable 3-13	−0.377	0.142	0.003**
EGF-containing fibulin-like extracellular matrix protein 1	−0.347	0.120	0.007**
Fibulin-1	−0.308	0.095	0.017*
Complement C1q subcomponent subunit C	−0.287	0.083	0.026*
Immunoglobulin heavy constant gamma 3	−0.274	0.075	0.034*
L-selectin	−0.273	0.074	0.035*
Vasorin	−0.261	0.068	0.044*

Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Table 3.9 *Significant correlations between HDL-associated proteins and ABCA1-dependent efflux.*

HDL-associated proteins	r	r^2	*P* value
Positively correlated proteins			
Immunoglobulin lambda-like polypeptide 1	0.384	0.148	0.003**
Kininogen-1	0.343	0.117	0.009**
Apolipoprotein B-100	0.331	0.109	0.012*
Vitamin K-dependent protein C	0.293	0.086	0.027*
CD5 antigen-like	0.286	0.082	0.031*
Negatively correlated proteins			
Complement C1q subcomponent subunit B (Fragment)	−0.389	0.151	0.003**
Vasorin	−0.332	0.11	0.012*
Fibulin-1	−0.324	0.105	0.014*
Immunoglobulin heavy variable 3-13	−0.309	0.095	0.019*
Immunoglobulin lambda variable 3-10	−0.308	0.095	0.020*
Sex hormone-binding globulin	−0.301	0.09	0.023*
Immunoglobulin heavy variable 3-64D	−0.299	0.089	0.024*
Hemoglobin subunit alpha	−0.27	0.073	0.042*
Hemoglobin subunit beta	−0.265	0.07	0.046*
L-selectin	−0.265	0.07	0.046*

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3.10 *Significant correlations between HDL-associated proteins and ABCA1-independent efflux.*

HDL-associated proteins	r	r^2	*P*value
Positively correlated proteins			
Apolipoprotein C-IV	0.399	0.159	0.002**
Apolipoprotein A-I	0.370	0.137	0.004**
Glutathione peroxidase	0.351	0.123	0.006**
Serum paraoxonase/arylesterase 1	0.332	0.110	0.0096*
Angiotensinogen	0.306	0.094	0.017*
Vitamin K-dependent protein C	0.306	0.093	0.018*
Apolipoprotein A-IV	0.305	0.093	0.018*
Endoplasmic reticulum chaperone BIP	0.298	0.089	0.021*
Beta-Ala-His dipeptidase	0.295	0.087	0.022*
Dopamine beta-hydroxylase	0.263	0.069	0.042*
Apolipoprotein C-III	0.26	0.068	0.045*
Negatively correlated proteins			
Immunoglobulin heavy constant gamma 3	−0.408	0.165	0.001**
EGF-containing fibulin-like extracellular matrix protein 1	−0.370	0.137	0.004**
Immunoglobulin heavy constant alpha 1	−0.364	0.132	0.004**
Complement C1q subcomponent subunit C	−0.321	0.103	0.012*
Immunoglobulin heavy variable 3-13	−0.306	0.094	0.017*
Immunoglobulin kappa constant	−0.282	0.079	0.029*
Probable non-functional immunoglobulin heavy variable 3-38	−0.276	0.076	0.033*
Complement C1q subcomponent subunit B (Fragment)	−0.269	0.072	0.038*

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

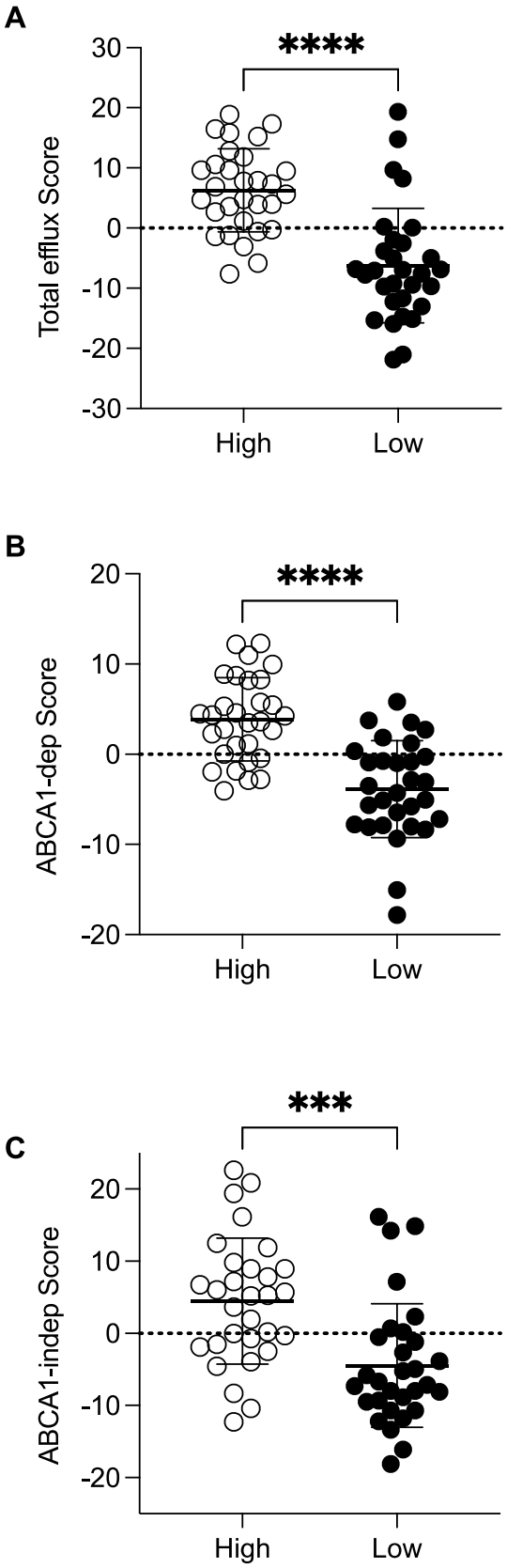


Figure 3.16 *HDL-CEC scores of participants with high and low HDL-CEC.*
HDL-CEC scores were developed using the significantly correlated HDL-associated proteins for each HDL-CEC parameter in participants with high (*n*=30) and low (*n*=30) efflux. Dot plots show results for **(A)** total efflux score, **(B)** ABCA1-dependent score, and **(C)** ABCA1-independent score. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

3.3.8 HDL-CEC Scores – correlations to metabolic health parameters, and univariable and multivariable associations

The total efflux score significantly positively correlated with total efflux (r=0.670, *P*<0.0001****), ABCA1-dependent efflux (r=0.549, *P*<0.0001****) and ABCA1-independent efflux (r=0.499, *P*<0.0001****) (**Table *3.11*; Figure 3.17 A**) and was a stronger predictor of total efflux than any given individual protein. The ABCA1-dependent score also significantly positively correlated with total efflux (r=0.668, *P*<0.0001****), ABCA1-dependent efflux (r=0.676, *P*<0.0001****) and to a lesser extent ABCA1-independent efflux (r=0.308, *P*=0.017*) (**Table *3.11*; Figure 3.17 B**). The ABCA1-independent score significantly correlated with total efflux (r=0.455, *P*=0.003***) and ABCA1-independent efflux (r=0.626, *P*<0.0001****) but not ABCA1-dependent efflux (r=0.179, *P*=0.173) (**Table *3.11*; Figure 3.17 C**). A significant correlation between HDL-associated ApoA-I and the total efflux score (r=0.269, *P*<0.037*), and ABCA1-independent score (r=0.530, *P*<0.0001****) was evident, with no association to the ABCA1-dependent score (r=−0.014, *P*=0.918) (**Table *3.11***).

There were no significant associations between the efflux scores and lipid parameters, with the exception of HDL-C, which was positively correlated with the ABCA1 independent score (**Table *3.11*** and **Table *3.14*** univariable analysis only).

In univariable and multivariable analysis, all three scores were positively associated with age. In univariable analysis, the total efflux score was negatively associated with smoking but this effect was lost in the multivariable analysis. In the multivariable analysis BMI was negatively associated with the total efflux score (**Table *3.12***).

Table 3.11 *Correlations of clinical and biochemical variables with HDL-CEC scores.*

Characteristics	r	r^2	*P* value
Total efflux Score			
Age (years)	0.385	0.032	0.0024*
Body mass index (kg/m^2)	−0.195	0.136	0.136
Systolic blood pressure (mm Hg)	0.093	0.009	0.508
Diastolic blood pressure (mm Hg)	0.076	0.006	0.591
Haemoglobin A1c (%)	0.020	0.0004	0.890
Total cholesterol (mmol/l)	0.270	0.073	0.053
Triglycerides (mmol/l)	0.123	0.015	0.380
LDL cholesterol (mmol/l)	0.078	0.006	0.587
HDL cholesterol (mmol/l)	0.226	0.051	0.107
Total efflux (%CEC/4h)	0.670	0.448	<0.0001****
ABCA1-dependent efflux (%CEC/4h)	0.549	0.301	<0.0001****
ABCA1-independent efflux (%CEC/4h)	0.499	0.249	<0.0001****
ABCA1-dependent score	0.760	0.578	<0.0001****
ABCA1-independent score	0.797	0.636	<0.0001****
ApoA-I	0.269	0.073	0.037*
ABCA1-dependent Score			
Age (years)	0.339	0.115	0.008**
Body mass index (kg/m^2)	0.114	0.013	0.385
Systolic blood pressure (mm Hg)	0.325	0.106	0.018*
Diastolic blood pressure (mm Hg)	0.232	0.054	0.095
Haemoglobin A1c (%)	0.256	0.066	0.079
Total cholesterol (mmol/l)	0.282	0.079	0.043*
Triglycerides (mmol/l)	0.293	0.086	0.033*
LDL cholesterol (mmol/l)	0.210	0.044	0.139
HDL cholesterol (mmol/l)	−0.103	0.01	0.469
Total efflux (%CEC/4h)	0.668	0.446	<0.0001****
ABCA1-dependent efflux (%CEC/4h)	0.676	0.456	<0.0001****
ABCA1-independent efflux (%CEC/4h)	0.308	0.095	0.017*
Total efflux score	0.760	0.578	<0.0001****
ABCA1-indepedndent score	0.519	0.269	<0.0001****
ApoA-I	−0.014	0.0002	0.918
ABCA1-independent Score			
Age (years)	0.439	0.193	0.0004***
Body mass index (kg/m^2)	−0.212	0.045	0.104
Systolic blood pressure (mm Hg)	0.019	0.0003	0.891
Diastolic blood pressure (mm Hg)	−0.045	0.002	0.749
Haemoglobin A1c (%)	0.163	0.027	0.268

Total cholesterol (mmol/l)	0.197	0.039	0.163
Triglycerides (mmol/l)	0.114	0.013	0.419
LDL cholesterol (mmol/l)	−0.023	0.0005	0.874
HDL cholesterol (mmol/l)	0.285	0.081	0.041*
Total efflux (%CEC/4h)	0.455	0.207	0.0003***
ABCA1-dependent efflux (%CEC/4h)	0.179	0.032	0.173
ABCA1-independent efflux (%CEC/4h)	0.626	0.392	<0.0001****
Total efflux score	0.797	0.636	<0.0001****
ABCA1-dependent score	0.519	0.269	<0.0001****
ApoA-I	0.530	0.281	<0.0001****

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

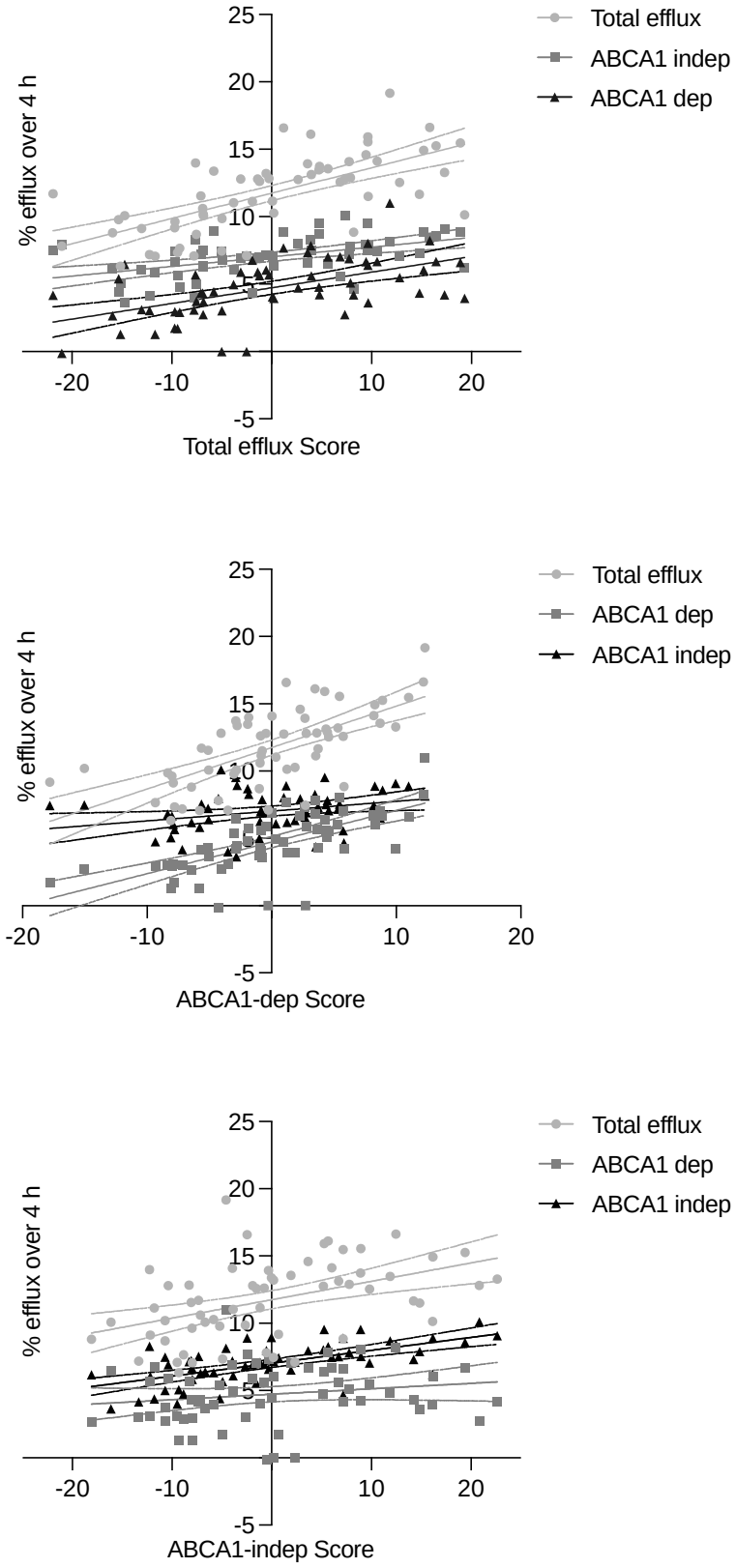


Figure 3.17 *Correlations of HDL-CEC Scores to HDL-CEC parameters.*
XY graphs show results for **(A)** total CEC score, **(B)** ABCA1-dependent CEC score, and **(C)** ABCA1-independent CEC score. Correlation lines are presented with 95% CI.

Table 3.12 *Univariable and multivariable association of clinical and biochemical variables with total CEC Score.*

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	0.264**	0.098, 0.43	0.593**	0.173, 1.013
Body mass index (kg/m^2)	−0.138	−0.320, 0.445	−0.374*	−0.656, −0.91
Sex (1 male; 2 female)	−0.008	−0.02, 0.004	3.341	−3.663, 10.345
Smoking (1 no; 2 yes)	−0.014**	−0.023, −0.004	−3.924	−9.871, 2.023
HDL cholesterol (mmol/l)	0.008	−0.002, 0.019	−1.657	−8.348, 5.033

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
β coefficient derived from linear regression and generalised estimating equation

Table 3.13 *Univariable and multivariable association of clinical and biochemical variables with ABCA1-dependent CEC Score.*

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	0.383**	0.104, 0.662	0.359**	0.107, 0.610
Body mass index (kg/m^2)	0.133	−0.171, 0.437	−0.042	−0.221, 0.137
Sex (1 male; 2 female)	−0.014	−0.034, 0.006	1.536	−3.309, 6.381
Smoking (1 no; 2 yes)	−0.011	−0.027, 0.005	−1.301	−5.236, 2.634
HDL cholesterol (mmol/l)	−0.006	−0.022, 0.01	−4.12	−8.415, 0.174

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
β coefficient derived from linear regression and generalised estimating equation

Table 3.14 *Univariable and multivariable association of clinical and biochemical variables with ABCA1-independent CEC Score.*

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	0.323***	0.150, 0.497	0.664**	0.243, 1.086
Body mass index (kg/m^2)	−0.161	−0.356, 0.032	−0.209	−0.553, 0.135
Sex (1 male; 2 female)	−0.012	−0.025, 0.001	1.83	−3.821, 7.48
Smoking (1 no; 2 yes)	−0.007	−0.018, 0.003	−1.747	−8.968, 5.474
HDL cholesterol (mmol/l)	0.011*	0.0005, 0.021	5.182	−2.724, 13.088

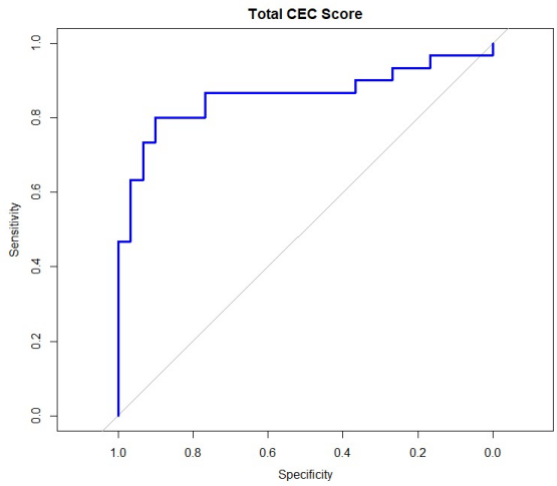
Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
β coefficient derived from linear regression and generalised estimating equation

3.3.9 Sensitivity and specificity of HDL-CEC Scores

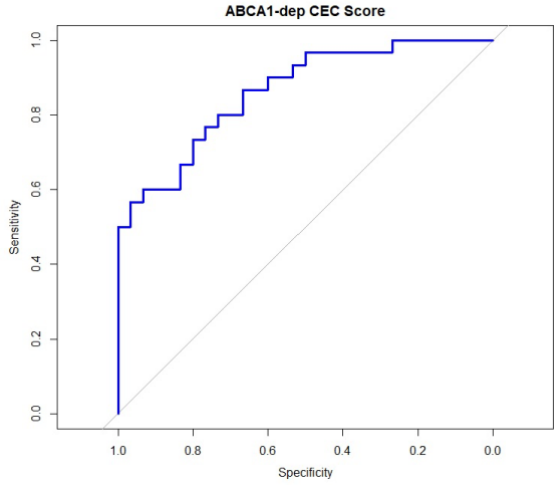
Receiver operating characteristic (ROC) curves were generated to assess the sensitivity and specificity for each of the HDL-CEC scores and are shown in **Figure 3.18 A-C**. The ROC curve is a plot of the true positive rate (TPR), or sensitivity, against the false positive rate (FPR), which is equivalent to 1-specificity, for all the possibly threshold/ cut-off points. The closer the curve is to the upper left corner the better the balance between the TPR and FPR. In cases where the model can perfectly distinguish between the groups, the TPR is equal to 1 and the FPR is equal to 0.

The total efflux score had the highest sensitivity (0.83) and specificity (0.80), while the ABCA1-independent score had the lowest sensitivity (0.77) and specificity (0.73) (**Figure 3.18 D**). The area under the curve (AUC) quantifies the overall ability of the test to discriminate between the two groups of high vs. low effluxers. A truly unfit test (one no better at identifying true positives than flipping a coin) has an area of 0.5, while a perfect test (one that has zero false positives and zero false negatives) has an area of 1.00. The AUC were 0.859 (*P*=0.0001***), 0.862 (*P*=0.0001***), and 0.781 (*P*=0.0002**) for the total efflux score, the ABCA1-dependent score and the ABCA1-independent score respectively (**Figure 3.18 D**).

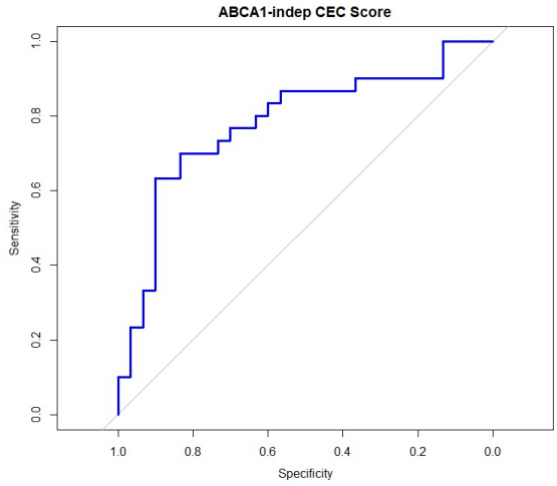
A



B



C



D

Score	Sensitivity	Specificity	AUC (*P* value)
Total CEC score	25/30=0.83	24/30=0.8	0.859 (*P*<0.0001)
ABCA1-dep score	24/30=0.8	23/30=0.77	0.862 (*P*<0.0001)
ABCA1-indep score	23/30=0.77	22/30=0.73	0.781 (*P*<0.0002)

Figure 3.18 *ROC curves comparing performance of HDL-CEC scores to differentiate between high vs. low effluxers.*
ROC curves show results for **(A)** total CEC score, **(B)** ABCA1-dependent CEC score, and **(C)** ABCA1-independent CEC score. Sensitivity and specificity are show in **(D).**

3.3.10 Total efflux score and ABCA1-dependent score are significantly different between high vs. low effluxers across all three groups

To identify whether the scoring algorithms also had predictive capacity with regards to HDL-CEC parameters in obesity and T2DM, the cohort was separated by presence of metabolic disease.

Between high vs. low HDL-CEC effluxers across all three groups there was a significant difference in the total efflux score and ABCA1-dependent score indicative that these scores are predictive of HDL-CEC in people with and without metabolic disease. However, while there was a significant difference in ABCA1-independent score between high vs. low HDL-CEC effluxers with metabolic disease, there was no significant difference in ABCA1-independent score between high vs. low HDL-CEC effluxers without metabolic disease, likely due to reduced power and/or proteins driving this score being modulated to a greater degree in participants with metabolic disease (**Figure 3.19**).

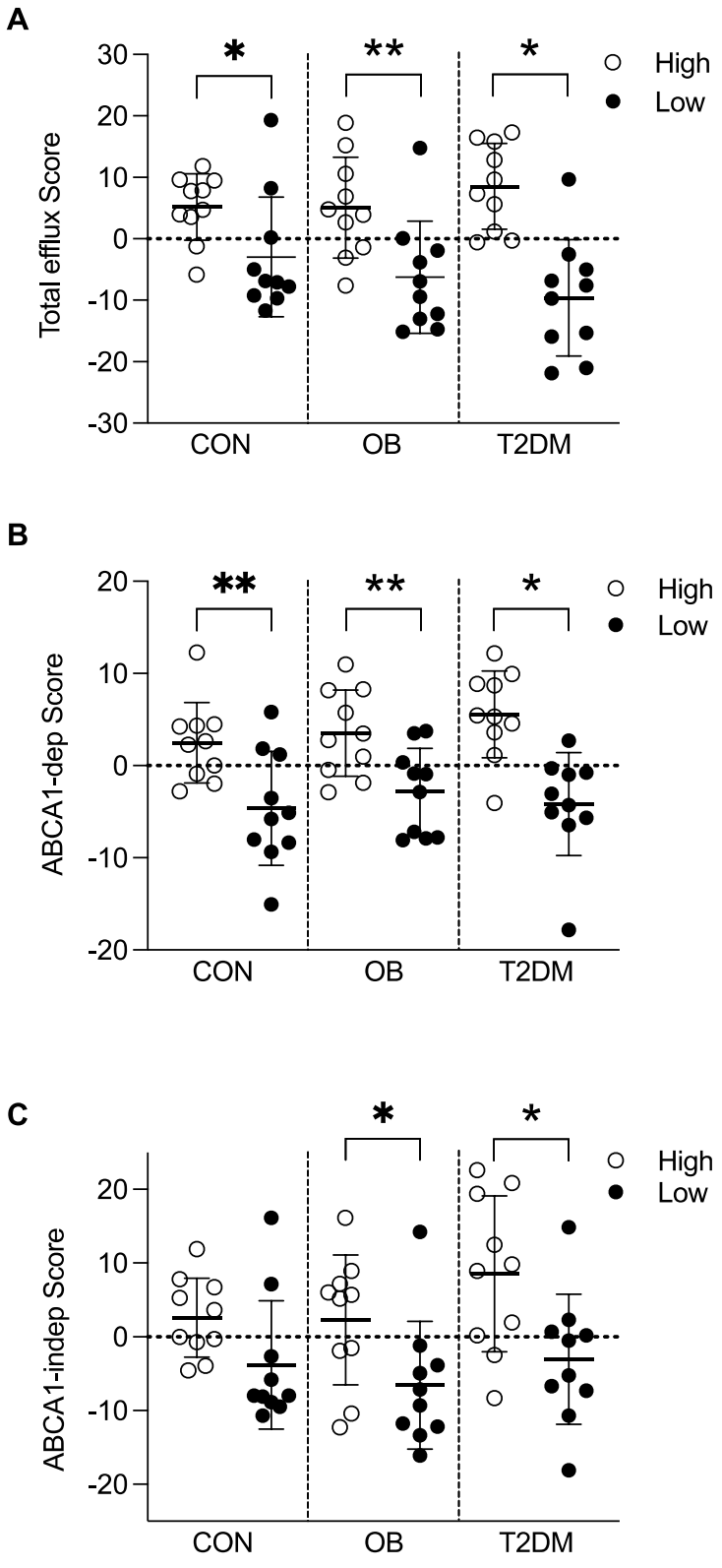


Figure 3.19 *HDL-CEC scores in participants with high and low HDL-CEC subdivided ±obesity and ±T2DM.*

HDL-CEC scores were developed using the significantly correlated HDL-associated proteins for each HDL-CEC parameter. Data was subdivided into Control (CON), Obesity (OB) and T2DM (*n*=10 per group). Dot plots show results for **(A)** total efflux score, **(B)** ABCA1-dependent score and, **(C)** ABCA1-independent score. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for high CEC vs. low CEC.

3.3.11 HDL-associated PON1 significantly correlated with total efflux but its activity does not

Given that HDL-associated PON1 protein levels appeared to be reduced in low effluxers with metabolic disease, we subsequently determined whether measuring serum PON1-activity, a traditional biomarker of the antioxidant function of HDL-P, might perform as a surrogate biomarker for HDL-CEC.

Serum PON1 activity was not significantly different between high HDL-CEC effluxers and low HDL-CEC effluxers. PON1 activity was also not significantly different when the cohort was separated by metabolic disease status (**Figure 3.20 A-B**).

HDL-associated PON1 significantly positively correlated with total CEC (r=0.273, *P*=0.035*) and ABCA1-independent CEC (r=0.332, *P*=0.0096**). It also significantly positively correlated with all three HDL-CEC scores; total efflux score (r=0.434, *P*=0.0005***), ABCA1-dependent score (r=0.325, *P*=0.011*), and ABCA1-independent score (r=0.519, *P*<0.0001****) (**Figure 3.20 C&E**).

However, serum PON1 activity did not significantly correlate with any HDL-CEC parameter; total efflux (r=0.060, *P*=0.662), ABCA1-dependent efflux (r=0.075, *P*=0.594) and ABCA1-independent efflux (r=0.044, *P*=0.750) or with any HDL-CEC score**;** total efflux score (r=0.114, *P*=0.405), ABCA1-dependent score (r=0.171, *P*=0.209) and ABCA1-independent score (r=0.105, *P*=0.444) (**Figure 3.20 D&F**). These findings indicate that direct measurement of HDL-associated PON1, and not PON1 activity, is a superior biomarker of HDL-CEC.

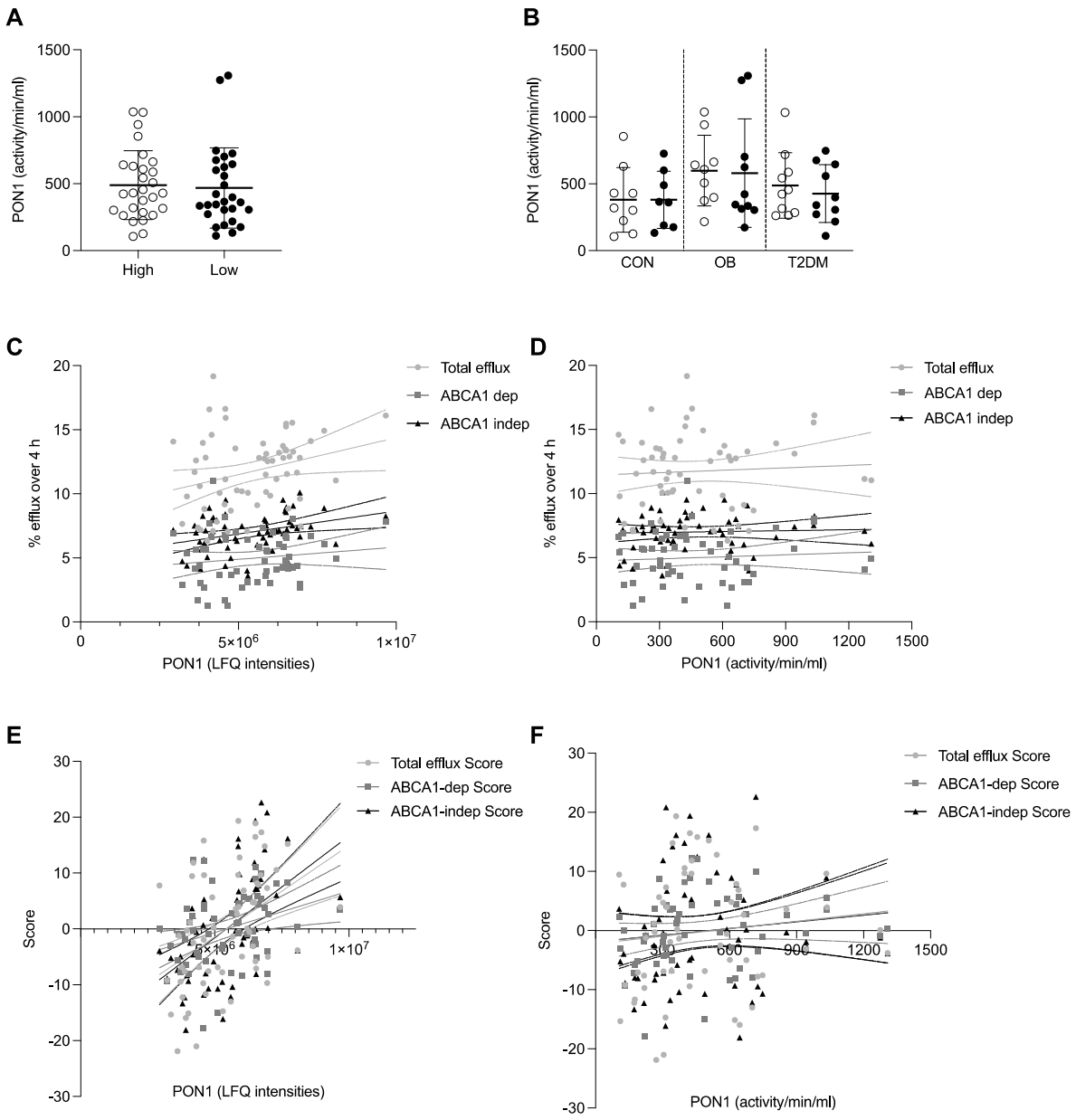


Figure 3.20 *HDL-associated PON1 and PON1 activity in high vs. low effluxers.*
Paraoxon ethyl was used to determine PON1 activity in **(A)** people with high HDL-CEC and low HDL-CEC (*n*=30 per group) and in **(B)** individuals subdivided into Control (CON), Obesity (OB) and T2DM (*n*=10 per group). Data are displayed as mean ± SD. Linear regression was conducted. XY graphs show results for **(C)** HDL-associated PON1 and HDL-CEC parameters, **(D)** PON1 activity and HDL-CEC parameters, **(E)** HDL-associated PON1 and HDL-CEC score, and **(F)** PON1 activity and HDL-CEC scores. Correlation lines are presented with 95% CI.

3.4 Discussion

Within this study we demonstrate, for the first time, that there are distinct HDL-protein footprints that can predict the total CEC of HDL-P, and furthermore distinct proteins associated with ABCA1-dependent and ABCA1-independent mediated efflux. We demonstrate that subtle differences in the HDL proteome are evident in low HDL effluxers relative to high HDL effluxers that collectively are associated with significant dysfunction of the HDL-P. HDL from low effluxers is enriched with fibulin and depleted of ApoC-III, ApoC-IV, platelet glycoprotein V and vitamin-K dependent protein C. We furthermore demonstrate that HDL-associated proteins, platelet glycoprotein V, histidine-rich glycoprotein and vitamin-K dependent protein C, are more markedly modulated in low-effluxers vs. high-effluxers with obesity/T2DM than those without metabolic disease, and may represent novel biomarkers of dysfunctional HDL and excess CVD risk in these individuals. We finally demonstrate that combining the proteins that significantly correlated with HDL-CEC, could generate three novel scores, total efflux score, ABCA1-dependent score, and ABCA1-independent score, that significantly correlated with total CEC (r=0.670, *P*<0.0001****), ABCA1-dependent CEC (r=0.676, *P*<0.0001****), and ABCA1-independent CEC (r=0.626 *P*<0.0001****) respectively. That withstanding, even with collation of the proteins into a score, the highest correlation evident to total CEC was r=0.670, indicative of residual CEC that is not governed by HDL-associated proteins. These findings have helped unravel the complex relationship between HDL-P, HDL-CEC and HDL protein composition, but have also exposed the complexity of this relationship with additional parameters beyond proteins likely playing a role such as particle size and/or lipid composition.

Measuring HDL-CEC within a cell-culture model has emerged as a novel target and more vital determinant of CVD risk than static HDL-C levels [7-9] however, a substantial challenge in realising the benefits of this measurement lies in the technical difficulties of conducting a standardised cell-based assay. There is a major unmet need to identify robust biomarkers of HDL-CEC in order to develop new more efficient methods of predicting incidence of and/or progression of CVD. HDL proteomics is one approach to biomarker discovery, which could enable large-scale studies with a method that is more suitable for clinical use.

A major obstacle for HDL proteomics is the lack of standardisation for the method of HDL isolation. Different isolation techniques may considerably effect the proteomic composition of HDL with a larger number of proteins typically identified following FPLC isolation relative to density gradient ultracentrifugation (DGUC). DGUC is traditionally considered the gold-standard for HDL isolation, however isolation via FPLC provides an alternative, reproducible method that results in a non-destructive separation of lipoproteins, and requires less serum sample [26]. The primary disadvantage of separating by FPLC is the overlap in size between

HDL and many high abundant plasma proteins that are assumed to be contaminants, in particular immunoglobulins (Igs). We therefore incorporated an LRA step following FPLC isolation, which enriches HDL fractions for phospholipid-containing particles, and depletes the resulting FPLC-HDL pellet of contaminants [27]. Regardless of which method is chosen, the central issue is to confirm that any predicted protein changes are functionally or clinically relevant [28].

Our study has identified changes in the profile of proteins associating with HDL in high HDL-CEC effluxers compared to low HDL-CEC effluxers (25 significantly changed proteins) with depletion of proteins involved in angiogenesis such as histidine-rich glycoprotein, proteins involved in blood coagulation such as platelet glycoprotein V and vitamin K-dependent protein C, and the antioxidant protein ApoA-IV on HDL from low effluxers as well as enrichment of proteins involved in the immune response and complement system such as C4b-binding protein beta chain on HDL from low effluxers. It is important to note however that changes in the levels of these HDL-associated proteins were subtle and it is therefore unlikely that any one protein is centrally involved in driving/ inhibiting efflux. – Indeed, Ig heavy variable 3-13 was the only protein to reach significance between high vs. low effluxers when accounting for multiple comparisons of all other proteins, and negatively correlated with all three CEC parameters. Hence, this immune protein may represent an important biomarker of low HDL-CEC. The strongest association between HDL-associated proteins and total CEC across the population was in the anticoagulant protein vitamin K-dependent protein C ($r=0.397$, $P=0.002$**) and in turn was the only protein to positively correlate with all three HDL-CEC parameters. Deficiency in this protein can lead to unchecked thrombin generation, and subsequently thromboembolism [29]. Hence, this protein could be one of the mediating factors implicated in the cardioprotective properties of HDL-P.

Within the study design we included individuals with obesity/T2DM to establish whether similar changes in HDL-protein footprints in high vs. low HDL-CEC effluxers were evident in participants with metabolic disease compared to those without metabolic disease, and to decipher whether our HDL-CEC scores would continue to perform in such a prevalent high-CVD risk population. A limitation of our approach is the relatively low *n*-numbers for this sub-analysis ($n=10$ per group). That withstanding, there were a number of notable observations – firstly the reductions of the aforementioned HDL-associated vitamin-K-dependent protein C and platelet glycoprotein V were more profound in low-effluxers with T2DM, relative to other groups. Vitamin K-dependent protein C may in turn represent an important biomarker of dysfunctional HDL in T2DM and represent a more stratified biomarker to determine CVD risk in this population. Platelet glycoprotein V functions as the von Willebrand Factor (vWF)

receptor and mediates vWF-dependent platelet adhesion to vascular damage and therefore plays an important role in clot formation [30]. The lack of functional platelet glycoprotein V on the platelet surface is the cause of a rare bleeding disorder associated with macro thrombocytopenia [30]. The role of this protein on HDL, however, is unknown but it may serve as another biomarker of dysfunctional HDL. Reduction in HDL-associated PON1 was also more pronounced in the low-effluxers with T2DM relative to high effluxers with T2DM, with minimal differences observed in the non-obese group. PON1 is a potent antioxidant protein, predominantly associated with HDL in serum [31]. PON1 has been shown to be involved in the atheroprotective properties of HDL preventing LDL from oxidative modification [32, 33]. Reductions in this HDL-associated PON1 in low effluxers with T2DM may further contribute to the increased residual CVD risk in these people. A significant reduction in HDL-associated histidine-rich glycoprotein, a protein with anticoagulant and antifibrinolytic activity [34], was only evident in low effluxers vs. high effluxers with obesity and T2DM, but not in normal-weight individuals, indicative that certain HDL-associated proteins may serve as specific biomarkers of low-efflux in people with metabolic disease. This is an important avenue for exploring in future validation studies and may proffer a novel stratified approach for CVD risk management in people with metabolic disease.

Another key observation of this study was the significant association between HDL-associated PON1 and total CEC and ABCA1-independent CEC. Given the relationship between HDL-associated PON1 and CEC, we thus speculated that measuring PON1 activity in serum may serve as a surrogate biomarker of HDL-CEC. Our findings, however, demonstrate that measurement of HDL-associated PON1 protein levels, and not serum PON1 activity, is a stronger predictor of CEC.

Correlations of HDL-CEC parameters to each other showed that total CEC had a stronger relationship with ABCA1-dependent efflux (r=0.856) compared to ABCA1-independent efflux (r=0.676), highlighting the importance of ABCA1-dependent efflux for removing cholesterol from cells. Within the current study, we found HDL-C to be positively associated with the ABCA1-independent efflux score within univariate analysis (but not ABCA1-dependent efflux score); however, this association was lost upon multivariate analysis. The reduced sample size within the current study likely contributed to lack of association between HDL-C and HDL-CEC parameters. Notably, age was a key variable that was positively associated with ABCA1-independent efflux in multivariate analysis, albeit in a relatively young/ middle-age population. It was also significantly positively associated with all HDL-CEC efflux scores. Previous findings in more elderly populations with and without CVD found no impact of age on HDL-CEC, but more marked changes in HDL proteomic composition [35]. Furthermore, in octogenarians,

HDL-CEC was found to be significantly higher (+30.2%) than healthy, younger, cardiovascular event-free middle aged individuals [36]. We previously reported a significant negative correlation between age, and total CEC as well as ABCA1-dependent efflux in T1DM [22]. Taken together, there is divergence in associations between age and HDL-CEC efflux parameters in different populations and the mechanisms at play are poorly understood. In the current study we also observed a significant inverse correlation between ABCA1-independent CEC and BMI in multivariable analysis. Similar negative associations between total CEC and BMI have been reported previously [8]; our findings highlight this negative association between BMI and total CEC is likely driven by reductions in ABCA1-independent efflux.

Surprisingly, ABCA1-dependent efflux, a key participant in the early acquisition of lipid to ApoA-I [37], did not significantly correlate with HDL-associated ApoA-I. However, the lipid poor preβ-1 HDL-P were likely not captured in the HDL-containing fractions (36-42) that were taken for proteomic analysis accounting for the discrepancies in our findings. It is more likely that these particles would co-elute with the serum peak during FPLC separation and thus be highly contaminated with serum proteins. It would be of interest in the future to use immunoprecipitation techniques to capture ApoA-I-containing particles in serum and analyse associated proteins and relationship to CEC [3, 38]. This would be an alternative approach to capture ApoA-I preβ-1 HDL-P.

To act as a surrogate biomarker for HDL-CEC, an expected biomarker would need to have a ROC curve with an AUC between 0.8-1 (with 0.5 indicating no discrimination and 1 indicating perfect discrimination) [39]. While we identified a number of associations between individual HDL-proteins and HDL-CEC, none reached a level that would be required for a stand-alone biomarker of HDL-CEC. We therefore created a scoring algorithm for each individual efflux parameter based on the proteins that significantly correlated with that given efflux parameter from the discovery proteomics results. We found that there are groups of proteins that can potentially differentiate between people with high and low HDL-CEC with separate groups of proteins predicting total CEC, ABCA1-dependent CEC, and ABCA1-independent CEC. Grouping the significantly correlated proteins for each HDL-CEC parameter to create a score resulted in stronger correlations to their respective HDL-CEC parameter than any individual protein alone; total efflux score to total efflux (r=0.670), ABCA1-dependent score to ABCA1-dependent efflux (r=0.676) and ABCA1-independent score to ABCA1-independent efflux (r=0.626). A correlation value of r>0.6 is considered a strong correlation [40]. Our ROC AUC in turn was 0.859, *P*=0.0001*** for total efflux score, 0.862, *P*=0.0001*** for ABCA1-dependent efflux score and 0.781, *P*=0.0002** for ABCA1-independent efflux score, indicating that our total efflux score and ABCA1-dependent score have the potential be surrogate biomarkers for

HDL-CEC, while the ABCA1-independent score requires further refinement. Our scores performed better than reported findings by Jin *et al.,* who developed a targeted proteomic panel and associated algorithm from the HDL-associated proteins, ApoA-I, ApoC-I, ApoC-II, ApoC-III and ApoC-IV and investigated the predictive capacity for both total CEC (ROC AUC=0.62) [41], and angiographically confirmed CAD (AUC=0.73). This score could also predict future risk of death from CAD, independent of circulating ApoA-I and ApoB concentrations and other conventional risk factors [42]. In a validation set of samples Jin *et al.,* found their efflux score performed even better (r=0.86, *P*<0.0001) [41]. However, the capacity of our score (ROC AUC=0.859) and findings by Jin *et al.,* (ROC AUC=0.62) indicates that there are residual factors at play that govern HDL-CEC beyond the HDL proteome. Notably, there were differences in approaches taken in both our respective studies from method of HDL isolation to proteomics analysis. Our study took a discovery proteomics approach on FPLC-derived HDL to identify novel proteins that associate with CEC, while Jin *et al.,* were restricted to a targeted proteomics analysis of five HDL-associated proteins in metal chelate affinity chromatography isolated HDL-P. We also expanded to investigate the relationship between the HDL proteome and HDL-CEC subtypes, including total, ABCA1-dependent and ABCA1-independent CEC, while Jin *et al.,* solely focused on total CEC. Further studies are needed to replicate and subsequently validate our findings in larger cohorts, and to determine if the efflux scores can predict future CVD events and therefore those most at risk of CVD.

The study includes the following strengths: (1) comparison between a cohort of 30 participants with high HDL-CEC carefully age, sex and BMI matched to 30 participants with low HDL-CEC participants; (2) profiling of the HDL proteome in 60 individuals; (3) detailed characteristics of participants including smoking status and medications; (4) no differences in the medications used between participants (5) analysis of total, ABCA1-dependent and ABCA1-independent CEC and (6) inclusion of individuals with and without metabolic disease. An important limitation of this study is the inability to make causal inference. This study was cross-sectional; therefore, it was not designed to determine the longitudinal effect of high vs. low HDL-CEC on incidence or progression of CVD, which would be needed to validate the predictive capacity of the HDL-CEC scores generated. While all groups were age, sex and BMI-matched, there was an increased prevalence of individuals who smoke within the low effluxer group, which may have causally impacted HDL-CEC. While cigarette smoking is associated with reduced HDL-C levels [43], the significant increased prevalence of smokers in the low effluxer group did not translate into a significant difference in HDL-C levels between the two groups. Smoking was significantly negatively associated with all HDL-CEC parameters in the univariable analysis. When adjusted for age, BMI, sex and HDL-C the association was lost in ABCA1-independent CEC, but retained for total and ABCA1-dependent CEC. Furthermore, smoking was not

associated with any of the HDL-CEC scores in the multivariable analysis, indicating that smoking did not affect our scores. The impact of smoking on HDL-CEC is controversial. Incubating HDL with cigarette smoke extract has been shown to modify HDL through lipid peroxidation and subsequently impair HDL-CEC *in vitro* [44]. Additionally, Khera *et al.,* one of the key studies investigating HDL-CEC, reported that current smoking was associated with decreased efflux capacity in a case-control study of 351 controls and 442 people with confirmed CAD [7]. In contrast, another key study, Saleheen *et al.,* found no relationship between whether one had never smoked, ever smoked or currently smokes and HDL-CEC in a large population study of 1745 participants with CHD and 1749 control participants [8]. Therefore, there is disagreement in associations between smoking and HDL-CEC efflux and the mechanisms at play are poorly understood and need further investigation. Another potential limitation is that the HDL-CEC scores were created by combining people ±obesity and ±T2DM into one cohort which could implicate the generalizability of the study. However, our findings demonstrate that the HDL-CEC scores generated were applicable for individuals with and without metabolic disease and inclusion of participants with metabolic disease likely identified important biomarkers of dysfunctional HDL, including platelet glycoprotein V and vitamin-K dependent protein C, that may otherwise not have been identified. This study combined analysis of men and women (40%/60% respectively) with sex-matched controls, however, in the multivariable analysis being a woman was associated with a 1.37% significant increase in ABCA1-independent efflux. Additionally, we previously demonstrated sex-specific differences in HDL biology in type 1 diabetes mellitus (T1DM), whereby increased ABCA1-dependent and ABCA1-independent efflux is evident in men but not women relative to the control group [22]. Sex-specific differences exist in HDL biology, and therefore combining men and women into a single cohort may result in the loss of unique HDL proteome signatures associated with sex. Future studies should take this into account and analyse men and women separately. Finally, it is notable in this study that we correlated the total HDL proteome to efflux capacity of small (ABCA1-dependent efflux) and large (ABCA1-independent efflux) HDL-P. Future studies correlating the HDL proteome and HDL-CEC of individual HDL-containing FPLC fractions would provide even greater resolution into the relationship between HDL function and protein composition.

In conclusion, we have demonstrated that there are HDL-associated protein biomarkers, that when combined in a scoring algorithm, strongly correlates with total CEC, ABCA1-dependent CEC, and ABCA1-independent CEC in a cross-sectional population. These findings indicate an important role for the HDL proteome in governing HDL-CEC. However there is residual CEC that is likely dictated by other measures of this complex particle's composition not captured in the proteomics efflux scores including lipidomic composition, particle size, post-

translational lipid modifications and/or post-translational protein modifications which requires further investigation. Unravelling the molecular determinants of HDL-mediated CEC may help the development of novel, standardised and sensitive biomarkers of HDL function that can replace cell-based assays.

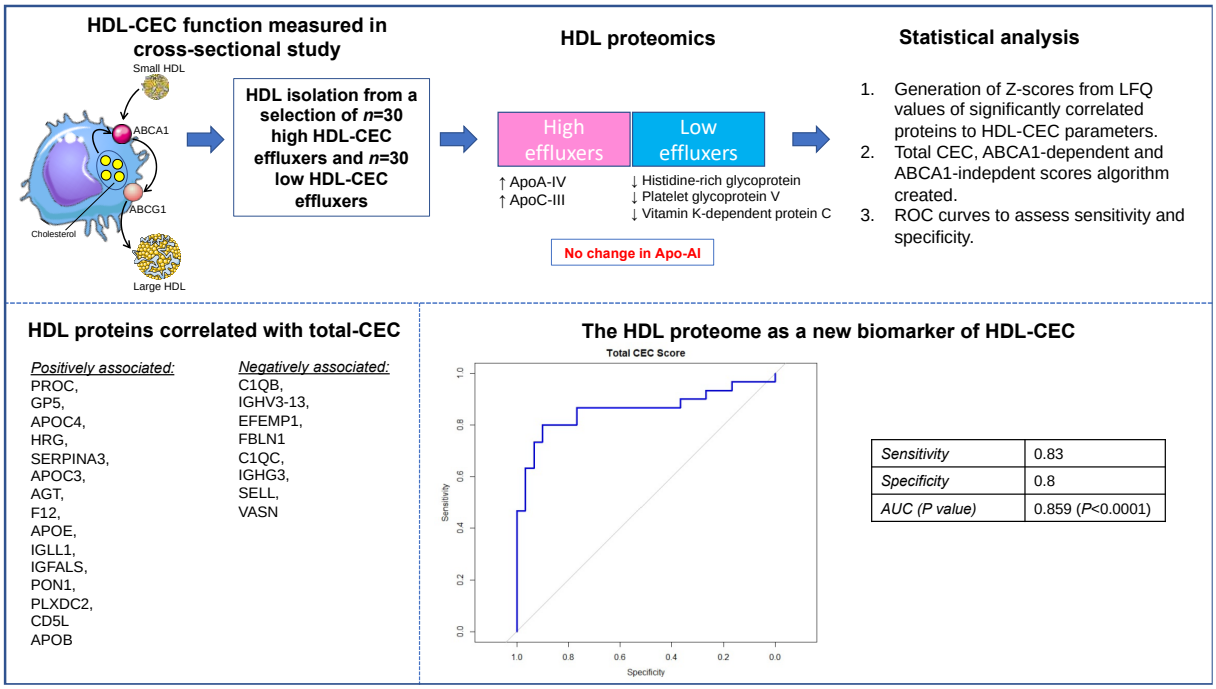


Figure 3.21 *Overall findings of chapter/Graphical abstract.*

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Appendix 3A – Medication table

Table 3.15 *Medication use in the high HDL-CEC and low HDL-CEC cohorts.*

	High HDL-CEC (*n*=30)	Low HDL-CEC (*n*=30)	
	%	**%**	*P* value
Medication use overall %	30	30	1.0
Medication			
ACE-I/ARB (anti-hypertensive)	0	6.6	0.274
Gliclazide (insulin-sensitising)	3.3	6.6	0.311
DPP4 inhibitor (insulin-sensitising)	6.6	0	0.140
Metformin (insulin-sensitising)	30	26.6	0.068
GLP-1	16.6	6.6	0.178
Statin	6.6	3.3	0.548

Differences between groups assessed using χ^2 test
People on medication were all people with T2DM

Appendix 3B – NCEP-ATP III criteria

Table 3.16 *National Cholesterol Education Program – Adult Treatment Panel III (NCEP-ATP III) criteria for Metabolic syndrome (MetS) [1].*

Risk Factor	Guideline
Waist Circumference	Men > 102 cm
	Women > 88 cm
Triglycerides	≥ 150 mg/dL
HDL-C	Men < 40 mg/dL
	Women < 50 mg/dL
Fasting Blood Glucose	≥ 100 mg/dL
Blood Pressure	≥ 130/≥ 85 mmHg

Presences of ≤2 indicates MHO, while ≥3 indicates MUO

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Appendix 3C – All proteins detected on HDL

225 proteins were detected on HDL and are shown below.

Table 3.17 *Proteins detected on HDL-P from high and low HDL-CEC effluxers.*

Protein names	Majority protein IDs	High HDL-CEC		Low HDL-CEC	
	Uniprot	Mean LFQ Intensity	SD LFQ Intensity	Mean LFQ Intensity	SD LFQ Intensity
72 kDa type IV collagenase	P08253	3.43E+04	8.47E+03	3.28E+04	1.01E+04
Actin, cytoplasmic 1	P60709	1.84E+05	6.45E+04	2.09E+05	1.47E+05
Afamin	P43652	1.45E+07	3.06E+06	1.48E+07	2.78E+06
Albumin	P02768	3.37E+08	8.96E+07	3.39E+08	1.57E+08
Alpha-1-acid glycoprotein 1	P02763	6.11E+04	4.28E+04	4.60E+04	5.85E+04
Alpha-1-acid glycoprotein 2	P19652	2.22E+05	1.03E+05	1.97E+05	8.75E+04
Alpha-1-antichymotrypsin	P01011	1.69E+07	5.07E+06	1.50E+07	4.74E+06
Alpha-1-antichymotrypsin	G3V3A0	1.23E+05	9.44E+04	7.39E+04	6.60E+04
Alpha-1-antitrypsin	P01009	9.37E+06	9.52E+06	8.14E+06	8.47E+06
Alpha-1B-glycoprotein	P04217	1.13E+07	3.33E+06	1.04E+07	3.25E+06
Alpha-2-antiplasmin	P08697	3.75E+06	1.09E+06	3.93E+06	1.22E+06
Alpha-2-HS-glycoprotein	P02765	7.82E+06	2.49E+06	6.97E+06	2.71E+06
Alpha-2-macroglobulin	P01023	3.67E+06	1.09E+06	3.42E+06	9.79E+05
Angiotensinogen	P01019	1.69E+06	6.08E+05	1.35E+06	4.96E+05
Antithrombin-III	P01008	1.67E+06	8.90E+05	2.02E+06	3.60E+06
Apolipoprotein A-I	P02647	1.83E+08	4.77E+07	1.67E+08	2.81E+07
Apolipoprotein A-II	V9GYG9	1.40E+07	3.46E+06	1.27E+07	2.70E+06
Apolipoprotein A-IV	P06727	1.22E+07	6.28E+06	9.62E+06	6.53E+06
Apolipoprotein B-100	P04114	8.26E+05	2.16E+05	7.74E+05	1.71E+05
Apolipoprotein C-I	K7ERI9	2.41E+06	1.01E+06	2.04E+06	8.30E+05
Apolipoprotein C-II	K7ER74	4.60E+05	1.61E+05	4.12E+05	2.95E+05
Apolipoprotein C-III	P02656	4.27E+06	1.90E+06	3.30E+06	1.68E+06
Apolipoprotein C-IV	P55056	1.90E+05	8.65E+04	1.30E+05	9.12E+04
Apolipoprotein D	P05090	4.38E+06	1.30E+06	4.37E+06	1.14E+06
Apolipoprotein E	P02649	3.95E+06	2.51E+06	3.23E+06	1.01E+06
Apolipoprotein L1	O14791	8.05E+05	3.60E+05	7.54E+05	2.56E+05
Apolipoprotein M	O95445	2.52E+06	4.44E+05	2.55E+06	5.42E+05
Attractin	O75882	5.39E+05	1.90E+05	6.04E+05	2.47E+05
Beta-2-glycoprotein 1	P02749	1.44E+06	1.87E+06	1.30E+06	2.28E+06
Beta-Ala-His dipeptidase	Q96KN2	1.27E+06	4.67E+05	1.03E+06	3.82E+05
Biotinidase	P43251	3.55E+05	1.58E+05	4.26E+05	1.82E+05
C3/C5 convertase	B4E1Z4	3.24E+07	5.92E+06	3.15E+07	5.11E+06
C4b-binding protein alpha chain	P04003	3.95E+05	1.55E+05	5.45E+05	3.82E+05
Cadherin-13	P55290	4.85E+04	1.59E+04	5.21E+04	1.71E+04
Cadherin-5	P33151	1.48E+05	6.66E+04	1.36E+05	5.57E+04
Carboxypeptidase N catalytic chain	P15169	9.92E+04	4.13E+04	1.06E+05	2.96E+04
Carboxypeptidase N subunit 2	P22792	3.39E+05	1.32E+05	3.15E+05	1.09E+05
Cartilage acidic protein 1 (Fragment)	Q5T4F6	2.84E+04	1.36E+04	2.34E+04	1.71E+04

Cartilage oligomeric matrix protein	G3XAP6	1.11E+05	4.90E+04	1.16E+05	5.08E+04
Catalase	P04040	1.27E+05	2.76E+05	1.50E+05	2.50E+05
CD5 antigen-like	O43866	9.51E+04	9.74E+04	5.81E+04	3.85E+04
Ceruloplasmin	P00450	7.64E+07	1.62E+07	7.60E+07	1.61E+07
Clusterin	P10909	1.83E+07	3.45E+06	1.89E+07	4.18E+06
Coagulation factor IX	P00740	7.45E+05	2.41E+05	7.22E+05	2.28E+05
Coagulation factor V	P12259	2.33E+05	9.99E+04	2.33E+05	9.07E+04
Coagulation factor X	P00742	1.74E+06	3.70E+05	1.65E+06	3.56E+05
Coagulation factor XII	P00748	5.27E+05	3.01E+05	3.68E+05	2.71E+05
Coagulation factor XIII B chain	P05160	3.32E+06	6.71E+05	3.17E+06	6.11E+05
Collagen alpha-3(VI) chain	P12111	4.00E+04	1.54E+04	4.57E+04	2.17E+04
Complement C1q subcomponent subunit B (Fragment)	A0A0A0MSV6	5.53E+04	4.02E+04	1.02E+05	9.50E+04
Complement C1q subcomponent subunit C	P02747	1.03E+05	5.21E+04	1.62E+05	1.00E+05
Complement C1r subcomponent-like protein	Q9NZP8	3.09E+05	1.08E+05	3.40E+05	9.25E+04
Complement C1s subcomponent	P09871	6.30E+06	8.04E+05	6.36E+06	8.82E+05
Complement C2	P06681	5.10E+05	1.12E+05	4.88E+05	1.56E+05
Complement C3	P01024	3.61E+08	5.94E+07	3.61E+08	5.17E+07
Complement C4-A	P0C0L4	8.47E+07	1.86E+07	8.14E+07	1.80E+07
Complement C4-B	P0C0L5	4.79E+06	1.99E+06	5.29E+06	2.80E+06
Complement C5	P01031	2.39E+07	3.98E+06	2.30E+07	4.66E+06
Complement component C6	P13671	6.17E+06	1.04E+06	6.04E+06	7.75E+05
Complement component C7	P10643	7.56E+06	1.73E+06	8.44E+06	4.19E+06
Complement component C8 alpha chain	P07357	1.78E+06	5.86E+05	1.60E+06	4.29E+05
Complement component C8 beta chain	P07358	1.64E+06	4.95E+05	1.50E+06	4.07E+05
Complement component C8 gamma chain	P07360	1.47E+06	4.75E+05	1.24E+06	3.48E+05
Complement component C9	P02748	3.64E+06	1.38E+06	3.29E+06	1.14E+06
Complement factor H	P08603	3.60E+06	6.15E+05	3.85E+06	7.88E+05
Complement factor H-related protein 1	Q03591	1.95E+06	7.20E+05	1.84E+06	7.54E+05
Complement factor H-related protein 2	P36980	4.16E+05	1.70E+05	4.68E+05	2.56E+05
Complement factor I	G3XAM2	2.38E+06	6.81E+05	2.17E+06	5.50E+05
Complement subcomponent C1r	F5H2D0	7.81E+05	1.63E+05	7.92E+05	1.59E+05
Corticosteroid-binding globulin	P08185	9.02E+04	3.02E+04	7.78E+04	3.34E+04
Cysteine-rich secretory protein 3	J3KPA1	3.26E+04	1.60E+04	3.48E+04	2.96E+04
Dopamine beta-hydroxylase	P09172	7.34E+04	4.84E+04	7.67E+04	5.71E+04
EGF-containing fibulin-like extracellular matrix protein 1	Q12805	2.50E+05	8.53E+04	3.27E+05	1.44E+05
Endoplasmic reticulum chaperone BiP	P11021	1.20E+05	2.52E+04	1.14E+05	3.66E+04
Extracellular matrix protein 1	Q16610	6.79E+05	2.08E+05	7.23E+05	2.05E+05
Extracellular superoxide dismutase [Cu-Zn]	P08294	7.51E+04	1.89E+05	7.55E+04	1.20E+05
Fibrinogen alpha chain	P02671	7.99E+04	4.49E+04	7.66E+04	4.39E+04
Fibronectin	P02751	6.21E+05	2.56E+05	5.98E+05	3.15E+05
Fibulin-1	P23142	8.58E+05	1.99E+05	1.02E+06	2.69E+05
Fructose-bisphosphate aldolase	A0A3B3IS80	5.50E+04	4.78E+04	7.00E+04	1.18E+05
Fructose-bisphosphate aldolase A	P04075	1.85E+04	2.41E+04	2.26E+04	2.50E+04
Gelsolin	P06396	2.31E+06	7.99E+05	2.25E+06	1.65E+06
Glutathione peroxidase	A0A087X1J7	3.05E+05	7.57E+04	2.84E+05	7.36E+04

Haptoglobin	P00738	4.50E+07	5.66E+07	5.70E+07	5.34E+07
Haptoglobin-related protein	P00739	2.89E+05	1.18E+05	3.33E+05	2.42E+05
Hemoglobin subunit alpha	P69905	1.64E+06	1.62E+06	3.05E+06	4.52E+06
Hemoglobin subunit beta	P68871	2.05E+06	2.02E+06	3.80E+06	6.01E+06
Hemopexin	P02790	4.96E+07	1.07E+07	4.51E+07	9.43E+06
Heparin cofactor 2	P05546	4.96E+06	1.36E+06	4.27E+06	1.06E+06
Hepatocyte growth factor activator	Q04756	5.68E+05	1.93E+05	6.40E+05	2.32E+05
Hepatocyte growth factor-like protein	P26927	5.13E+04	3.31E+04	4.50E+04	2.55E+04
Histidine-rich glycoprotein	P04196	6.13E+06	2.21E+06	4.61E+06	2.11E+06
Hyaluronan-binding protein 2	Q14520	1.63E+05	1.16E+05	1.19E+05	9.42E+04
Ig-like domain-containing protein (Fragment)	A0A0J9YY99	2.24E+06	7.47E+05	2.27E+06	7.14E+05
Immunoglobulin heavy constant alpha 1	P01876	4.24E+07	1.46E+07	5.07E+07	1.67E+07
Immunoglobulin heavy constant alpha 2 (Fragment)	A0A0G2JMB2	5.66E+05	4.75E+05	8.09E+05	4.39E+05
Immunoglobulin heavy constant delta	P01880	1.21E+06	1.52E+06	1.02E+06	1.44E+06
Immunoglobulin heavy constant gamma 1 (Fragment)	A0A0A0MS08	1.77E+08	2.82E+07	1.78E+08	2.87E+07
Immunoglobulin heavy constant gamma 2	P01859	5.38E+07	1.96E+07	5.26E+07	1.78E+07
Immunoglobulin heavy constant gamma 3	P01860	5.95E+07	2.37E+07	6.78E+07	2.13E+07
Immunoglobulin heavy constant gamma 4	P01861	2.06E+07	1.32E+07	2.32E+07	2.27E+07
Immunoglobulin heavy constant mu	P01871	2.36E+06	1.45E+06	1.79E+06	8.04E+05
Immunoglobulin heavy variable 1-18	A0A0C4DH31	9.11E+05	5.04E+05	9.80E+05	3.68E+05
Immunoglobulin heavy variable 1-2	P23083	2.45E+06	1.09E+06	2.88E+06	1.21E+06
Immunoglobulin heavy variable 1-24	A0A0C4DH33	2.86E+05	1.87E+05	2.98E+05	1.96E+05
Immunoglobulin heavy variable 1-3	A0A0C4DH29	1.68E+06	7.96E+05	1.62E+06	8.74E+05
Immunoglobulin heavy variable 1-46	P01743	3.52E+05	2.60E+05	3.73E+05	4.11E+05
Immunoglobulin heavy variable 1-69D	A0A0B4J2H0	2.50E+05	1.65E+05	3.62E+05	2.62E+05
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	A0A075B7D0	5.07E+05	2.72E+05	4.65E+05	2.67E+05
Immunoglobulin heavy variable 2-26	A0A0B4J1V2	1.58E+05	9.93E+04	1.48E+05	6.96E+04
Immunoglobulin heavy variable 2-70D	A0A0C4DH43	1.14E+06	7.13E+05	1.08E+06	7.50E+05
Immunoglobulin heavy variable 3-13	P01766	1.19E+05	7.08E+04	2.15E+05	9.58E+04
Immunoglobulin heavy variable 3-15	A0A0B4J1V0	1.10E+06	6.49E+05	1.13E+06	4.11E+05
Immunoglobulin heavy variable 3-43	A0A0B4J1X8	1.62E+05	5.61E+04	1.69E+05	6.77E+04
Immunoglobulin heavy variable 3-49	A0A0A0MS15	1.73E+06	1.16E+06	1.84E+06	7.60E+05
Immunoglobulin heavy variable 3-64D	A0A0J9YX35	2.60E+05	1.41E+05	3.69E+05	2.92E+05
Immunoglobulin heavy variable 3-7	P01780	6.12E+06	2.61E+06	6.29E+06	2.04E+06
Immunoglobulin heavy variable 3-72	A0A4W8ZXM2	7.15E+06	2.76E+06	7.30E+06	2.66E+06
Immunoglobulin heavy variable 3-73	A0A0B4J1V6	1.41E+05	1.19E+05	1.66E+05	1.01E+05
Immunoglobulin heavy variable 3-74	A0A0B4J1X5	2.57E+06	1.65E+06	2.71E+06	1.51E+06
Immunoglobulin heavy variable 3-9	P01782	3.87E+05	2.24E+05	4.81E+05	3.40E+05
Immunoglobulin heavy variable 4-28	A0A0C4DH34	1.43E+06	4.71E+05	1.52E+06	5.94E+05
Immunoglobulin heavy variable 4-34	P06331	5.26E+06	1.10E+06	5.61E+06	1.73E+06
Immunoglobulin heavy variable 4-4	A0A075B6R2	3.73E+05	2.16E+05	4.28E+05	3.37E+05
Immunoglobulin heavy variable 5-51	A0A0C4DH38	3.58E+06	1.50E+06	3.97E+06	9.03E+05
Immunoglobulin heavy variable 6-1	A0A0B4J1U7	6.19E+05	3.41E+05	7.49E+05	7.23E+05
Immunoglobulin J chain	P01591	1.38E+05	7.47E+04	1.51E+05	6.28E+04

Immunoglobulin kappa constant	P01834	1.84E+08	3.58E+07	1.95E+08	3.56E+07
Immunoglobulin kappa variable 1-17	P01599	8.57E+05	4.09E+05	1.00E+06	5.82E+05
Immunoglobulin kappa variable 1-33	A0A2Q2TTZ9	6.92E+06	2.47E+06	6.97E+06	1.77E+06
Immunoglobulin kappa variable 1-5	P01602	1.91E+06	7.97E+05	2.08E+06	7.89E+05
Immunoglobulin kappa variable 1-8	A0A0C4DH67	7.79E+05	3.70E+05	8.14E+05	3.59E+05
Immunoglobulin kappa variable 1D-39	P04432	8.91E+04	6.67E+04	7.88E+04	7.53E+04
Immunoglobulin kappa variable 2-24	A0A0C4DH68	4.49E+05	5.73E+05	3.70E+05	3.03E+05
Immunoglobulin kappa variable 2-30	P06310	3.51E+06	1.10E+06	4.01E+06	9.44E+05
Immunoglobulin kappa variable 2-40	A0A087X0Q4	1.31E+06	4.17E+05	1.48E+06	6.27E+05
Immunoglobulin kappa variable 2D-28	A0A5H1ZRS2	1.94E+06	6.91E+05	2.03E+06	8.15E+05
Immunoglobulin kappa variable 2D-29	A0A5H1ZRS9	4.48E+05	4.28E+05	5.23E+05	3.92E+05
Immunoglobulin kappa variable 3-11	P04433	9.18E+05	5.55E+05	9.67E+05	3.17E+05
Immunoglobulin kappa variable 3-15	P01624	7.71E+04	5.12E+04	1.10E+05	7.41E+04
Immunoglobulin kappa variable 3-20	P01619	3.26E+06	1.11E+06	3.24E+06	1.06E+06
Immunoglobulin kappa variable 4-1	P06312	3.93E+06	2.09E+06	3.92E+06	1.28E+06
Immunoglobulin kappa variable 6D-21	A0A0A0MT36	4.19E+05	3.28E+05	4.06E+05	2.20E+05
Immunoglobulin lambda constant 2	P0DOY2	7.65E+04	5.96E+04	7.53E+04	4.23E+04
Immunoglobulin lambda constant 3	P0DOY3	9.42E+07	2.49E+07	1.02E+08	2.74E+07
Immunoglobulin lambda constant 7 (Fragment)	A0A5H1ZRQ7	6.46E+05	3.76E+05	6.72E+05	2.72E+05
Immunoglobulin lambda variable 1-40	P01703	1.50E+05	7.74E+04	1.70E+05	6.18E+04
Immunoglobulin lambda variable 1-47	P01700	5.69E+05	3.32E+05	6.16E+05	2.48E+05
Immunoglobulin lambda variable 1-51	P01701	3.08E+05	2.48E+05	2.97E+05	1.45E+05
Immunoglobulin lambda variable 2-11	P01706	3.35E+05	3.87E+05	2.41E+05	1.68E+05
Immunoglobulin lambda variable 2-14	P01704	1.78E+05	1.39E+05	1.77E+05	1.55E+05
Immunoglobulin lambda variable 2-18	A0A075B6J9	3.48E+05	5.05E+05	2.08E+05	1.76E+05
Immunoglobulin lambda variable 3-10	A0A075B6K4	1.56E+06	5.85E+05	1.82E+06	8.33E+05
Immunoglobulin lambda variable 3-19	P01714	5.10E+05	3.33E+05	5.45E+05	3.06E+05
Immunoglobulin lambda variable 3-21	P80748	2.54E+06	1.78E+06	3.15E+06	2.41E+06
Immunoglobulin lambda variable 3-25	P01717	7.59E+05	4.69E+05	8.19E+05	3.52E+05
Immunoglobulin lambda variable 4-69	A0A075B6H9	4.27E+05	2.16E+05	3.90E+05	1.42E+05
Immunoglobulin lambda variable 6-57	P01721	1.13E+05	8.28E+04	9.98E+04	7.36E+04
Immunoglobulin lambda variable 8-61	A0A075B6I0	1.16E+06	7.27E+05	9.61E+05	4.82E+05
Immunoglobulin lambda variable 9-49	A0A0B4J1Y8	1.83E+05	1.44E+05	1.76E+05	1.55E+05
Immunoglobulin lambda-like polypeptide 1	P15814	1.92E+05	1.42E+05	1.90E+05	1.62E+05
Immunoglobulin lambda-like polypeptide 5	B9A064	1.42E+07	7.49E+06	1.68E+07	5.83E+06
Insulin-like growth factor-binding protein 3	A6XND1	5.45E+05	1.77E+05	4.96E+05	1.47E+05
Insulin-like growth factor-binding protein complex acid labile subunit	P35858	3.78E+06	8.11E+05	3.40E+06	1.05E+06
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	5.94E+06	1.70E+06	5.77E+06	1.88E+06
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	1.12E+07	3.28E+06	1.15E+07	3.21E+06
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033	1.37E+06	6.09E+05	1.39E+06	5.24E+05
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	2.17E+07	5.24E+06	2.27E+07	4.38E+06
Intercellular adhesion molecule 1	P05362	3.79E+04	1.50E+04	4.09E+04	2.13E+04
ITIH4 protein	B7ZKJ8	6.51E+04	3.95E+04	6.57E+04	2.77E+04
Kallistatin	P29622	7.45E+04	4.06E+04	7.24E+04	5.64E+04
Keratin, type I cytoskeletal 10	P13645	1.68E+05	2.17E+05	1.33E+05	1.33E+05

Keratin, type I cytoskeletal 9	P35527	1.31E+05	1.40E+05	2.22E+05	3.10E+05
Keratin, type II cytoskeletal 1	P04264	4.85E+05	4.39E+05	4.84E+05	4.53E+05
Kininogen-1	P01042	2.34E+07	4.01E+06	2.16E+07	3.45E+06
L-lactate dehydrogenase B chain	P07195	5.61E+04	3.31E+04	5.75E+04	2.92E+04
L-selectin	P14151	4.43E+05	1.15E+05	4.73E+05	9.42E+04
Low affinity immunoglobulin gamma Fc region receptor III-A	P08637	2.44E+05	5.32E+04	2.58E+05	6.47E+04
Lumican	P51884	3.33E+06	5.18E+05	3.49E+06	7.98E+05
Macrophage colony-stimulating factor 1 receptor	P07333	9.75E+04	5.01E+04	1.06E+05	4.70E+04
Mannan-binding lectin serine protease 1	P48740	3.45E+05	1.32E+05	3.40E+05	1.17E+05
Mannan-binding lectin serine protease 2	O00187	1.02E+05	4.26E+04	8.16E+04	3.37E+04
Membrane primary amine oxidase	Q16853	3.45E+04	1.13E+04	3.34E+04	1.06E+04
N-acetylmuramoyl-L-alanine amidase	Q96PD5	1.59E+06	3.80E+05	1.58E+06	3.92E+05
Neuropilin	E9PEP6	7.48E+04	2.78E+04	7.59E+04	2.59E+04
Phosphatidylcholine-sterol acyltransferase	P04180	4.77E+05	1.28E+05	4.55E+05	1.33E+05
Phosphatidylinositol-glycan-specific phospholipase D	P80108	1.29E+06	4.53E+05	1.33E+06	5.11E+05
Phospholipid transfer protein	P55058	7.95E+04	2.17E+04	8.00E+04	2.03E+04
Plasma kallikrein	P03952	4.56E+06	1.46E+06	4.43E+06	1.23E+06
Plasma protease C1 inhibitor	P05155	7.53E+06	2.73E+06	8.57E+06	2.73E+06
Plasminogen	P00747	1.06E+06	4.91E+05	9.40E+05	8.99E+05
Platelet glycoprotein V	P40197	5.72E+04	3.48E+04	3.13E+04	2.28E+04
Plexin domain-containing protein 2	Q6UX71	6.79E+04	2.05E+04	5.53E+04	1.98E+04
Pregnancy zone protein	P20742	1.12E+05	9.28E+04	1.08E+05	1.44E+05
Prenylcysteine oxidase 1	Q9UHG3	1.42E+05	6.40E+04	1.47E+05	4.91E+04
Probable non-functional immunoglobulin heavy variable 3-35	A0A0C4DH35	6.37E+05	2.79E+05	5.89E+05	3.38E+05
Probable non-functional immunoglobulin heavy variable 3-38	A0A0C4DH36	6.14E+05	4.86E+05	5.69E+05	4.02E+05
Probable non-functional immunoglobulin heavy variable 3-38-3	P0DTE1	1.69E+05	6.68E+04	1.65E+05	6.25E+04
Probable non-functional immunoglobulin kappa variable 3-7	A0A075B6H7	9.10E+05	4.95E+05	9.57E+05	4.15E+05
Properdin	P27918	6.13E+04	2.43E+04	7.93E+04	3.02E+04
Protein AMBP	P02760	4.08E+06	1.12E+06	4.40E+06	1.26E+06
Protein Z-dependent protease inhibitor	Q9UK55	5.82E+05	1.50E+05	5.63E+05	1.52E+05
Prothrombin	P00734	1.08E+07	3.38E+06	1.09E+07	4.67E+06
SAA2-SAA4 readthrough	A0A096LPE2	2.94E+06	1.06E+06	2.95E+06	8.41E+05
Selenoprotein P (Fragment)	A0A182DWH7	5.71E+05	1.93E+05	5.32E+05	2.08E+05
Serotransferrin	P02787	3.04E+07	1.12E+07	3.14E+07	1.56E+07
Serum amyloid A-1 protein	P0DJI8	9.47E+04	8.08E+04	8.36E+04	7.97E+04
Serum amyloid P-component	P02743	1.45E+06	6.76E+05	1.68E+06	6.00E+05
Serum paraoxonase/arylesterase 1	P27169	5.77E+06	1.43E+06	5.10E+06	1.26E+06
Serum paraoxonase/lactonase 3	Q15166	2.11E+05	9.55E+04	2.00E+05	8.70E+04
Sex hormone-binding globulin	I3L145	2.11E+05	1.49E+05	2.33E+05	1.33E+05
SPARC	P09486	1.76E+05	4.47E+04	1.67E+05	6.10E+04
Sulfhydryl oxidase 1	O00391	1.90E+05	4.33E+04	2.05E+05	6.89E+04
Tetranectin	E9PHK0	2.51E+06	5.43E+05	2.56E+06	4.78E+05
Thyroxine-binding globulin	P05543	1.04E+05	5.67E+04	1.01E+05	3.88E+04

Transferrin receptor protein 1	P02786	2.28E+05	8.91E+04	2.12E+05	1.12E+05
Transforming growth factor-beta-induced protein ig-h3	Q15582	4.83E+05	8.91E+04	4.55E+05	1.44E+05
Transthyretin	P02766	9.92E+04	1.09E+05	8.71E+04	1.73E+05
Vasorin	Q6EMK4	6.99E+04	2.17E+04	8.14E+04	2.34E+04
Vitamin D-binding protein	P02774	4.78E+05	1.38E+05	6.51E+05	9.22E+05
Vitamin K-dependent protein C	P04070	5.32E+05	1.21E+05	4.43E+05	1.19E+05
Vitamin K-dependent protein S	A0A3B3ISJ1	1.39E+06	3.01E+05	1.30E+06	3.08E+05
Vitamin K-dependent protein Z	P22891	4.65E+04	2.00E+04	5.14E+04	3.58E+04
Vitronectin	P04004	1.24E+07	3.39E+06	1.32E+07	3.47E+06
von Willebrand factor	P04275	5.44E+04	3.00E+04	6.26E+04	1.92E+04

Chapter 4

Investigating the impact of type 1 diabetes mellitus and type 2 diabetes on HDL function, and composition in humans.

Rachel Byrne, Eugene Dillon, Seán Curley, Mohamed Ahmed, Khalid Ahmed, Anne McGowan, Anjuli Gunness, Isolda Frizelle, Agnieszka Pazderska, Andrew Hogan, Donal O'Shea, James Gibney, and Fiona McGillicuddy.

Abstract

Introduction: The mechanisms underpinning increased CVD risk in people with T1DM and T2DM are poorly understood. The cardioprotective properties of HDL-particles (HDL-P), including antioxidant, anti-inflammatory, and cholesterol efflux capacity (CEC), align with assigned functions of the HDL proteome. Within this study, we hypothesized that changes in HDL structure and proteomic composition contributes to HDL dysfunction in diabetes.

Methods: Individuals with T1DM or T2DM, were age-, sex-, and BMI-matched to respective controls (*n*=81 per group). Total, ABCA1-dependent, and ABCA1-independent efflux were determined after 4h incubation of PEG-supernatants with ^{3}H-cholesterol labeled J774 macrophages treated ± cAMP *ex vivo*. Serum samples were sent for NMR lipoprotein analysis to LabCorp (LipoScience, Inc), NC, USA. HDL protein composition was investigated by discovery proteomics after fast-protein liquid chromatography isolation and lipid removal agent enrichment of HDL-containing fractions, with subsequent separation of HDL into large (L-HDL) and small (S-HDL) sub-fractions (*n*=20 per group).

Results: Individuals with T1DM exhibited enhanced total HDL-CEC and ABCA1-dependent CEC and increased concentration of L-HDL-P relative to T2DM. L-HDL-P concentration correlated with total (r=0.425) and ABCA1-independent CEC (r=0.476) across all groups, while S-HDL-P concentration only positively correlated with ABCA1-dependent CEC in the T2DM group (r=0.412). In T1DM, ApoM on L-HDL-P was positively associated with ABCA1-independent efflux (r=0.718), while association of immunoglobulins (Igs) was a negative determinant of ABCA1-independent efflux. In T2DM, association of ApoE (r=0.613) and vitamin-K dependent protein C (r=0.649) on L-HDL-P were positively associated with ABCA1-dependent CEC while ApoC-II was a positive determinant of ABCA1-independent efflux (r=0.464). Finally, we demonstrate few associations between S-HDL-P size, or the proteome of S-HDL-P, with HDL-CEC, except for the T2DM group. Correlations between ApoC-I, ApoC-II, ApoC-III, and ApoE on S-HDL-P with total CEC (r=0.667, r=0.582, r=0.584 and r=0.481 respectively) and ABCA1-dependent CEC (r=0.633, r=0.495, r=0.640 and r=0.640 respectively) were evident in T2DM. A significant correlation between ApoA-I and ApoA-II on L-HDL-P with ABCA1-independent CEC was evident in both the non-obese (r=0.604 and r=0.630) and obese (r=0.515 and r=0.474) control groups; these associations were completely abrogated in both T1DM (r=0.165 and r=0.199) and T2DM (r=−0.085 and r=0.066).

Conclusions: Our findings demonstrate that HDL functionality and composition are differentially modulated in individuals with T1DM and T2DM and different patterns of proteins are associated with HDL-efflux capacity in these disease states. A notable loss in association between HDL-CEC and HDL-associated ApoA-I and ApoA-II was evident in the setting of

diabetes. Further research will be required to tease apart HDL-proteins causally associated with HDL dysfunction from those that are merely correlative.

Abbreviations

ABCA1	ATP-Binding Cassette Subfamily A Member 1
ABCG1	ATP-binding Cassette Subfamily G, member 1
ACE-I	Angiotensin-Converting Enzyme Inhibitors
ANOVA	Analysis of variance
Apo	Apolipoprotein
ApoA-I	Apolipoprotein A-I
ARB	Angiotensin Receptor Blockers
BMI	Body Mass Index
C3/C5	C3/C5 Convertase
cAMP	Cyclic Adenosine Monophosphate
CEC	Cholesterol Efflux Capacity
CETP	Cholesterol Ester Transfer Protein
CF1	Complement Factor 1
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
DPP4	Dipeptidyl Peptidase-4
FDR	False Discovery Rate
FPLC	Fast Protein Liquid Chromatography
GLP-1	Glucagon-like Peptide-1
HbA1c	Haemoglobin A1c
HDL	High-Density Lipoprotein
HDL-C	High-Density Lipoprotein-Cholesterol
HDL-P	High-Density Lipoprotein-Particle
Ig	Immunoglobulin
ITIH	Inter-Alpha-Trypsin Inhibitor Heavy Chain H
KLKB1	Plasma Kallikrein
LCAT	Lecithin-Cholesterol Acyltransferase/ Phosphatidylcholine-sterol Acyltransferase
LDL	Low-Density Lipoprotein
LDL-C	Low-Density Lipoprotein-Cholesterol
LFQ	Label-Free Quantification
LRA	Lipid Removal Agent
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
OB	Obesity/Obese

PON1	Paraoxonase 1/ Serum Paraoxonase/Arylesterase 1
PLTP	Phospholipid Transfer Protein
RCT	Reverse Cholesterol Transport
SAA1	Serum Amyloid A 1
SD	Standard Deviation
SHBG	Sex Hormone Binding Globulin
SR-B1	Scavenger Receptor Class B Type 1
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides

4.1 Introduction

The International Diabetes Federation estimate that one in ten adults have diabetes globally, and by 2045, there will be an estimated 783 million adults with diabetes [1]. Despite many improvements in the management of diabetes, it is still a cause of disability and premature death, and resulted in approximately 6.7 million deaths globally in 2021 in the 20-79 year age group [1]. Morbidity and mortality due to diabetes are mostly attributable to an elevated risk of cardiovascular disease (CVD), which accounts for between one-third and one-half of all deaths [2]. Across the full range of fasting glucose, haemoglobin A1c (HbA1c), or 2 hour glucose test results, each standard deviation (SD) is associated with a 6-20% increased risk of CVD [2].
While people with T1DM, and people with T2DM exhibit an increased risk for CVD, a major confounder in people with T2DM is the presence of obesity. Metabolic dyslipidaemia is a classic hallmark of obesity and T2DM, with increased levels of small, dense low-density lipoprotein particles and circulating triglycerides (TG), and reduced levels of high-density lipoprotein (HDL)-cholesterol (HDL-C) [3-6]. In contrast to people with T2DM, lipid profiles in T1DM are normal or even apparently better than the general population, with greater HDL-C levels and lower LDL-C, and TG levels [7]. Considering the differing lipid profiles between T1DM and T2DM, and that higher levels of LDL-C are associated with increased risk of CVD [8], while HDL-C is negatively associated with CVD [9], it is likely the underlying mechanisms driving CVD are different for both diseases. However, the data are limited, and head-to-head comparisons of these two disease states are lacking.

In the clinical setting, standard lipid analysis includes measurement of fasting serum or plasma total cholesterol, TG, HDL-C, and LDL-C [10]. However, these relatively simple lipid measurements potentially mask more subtle lipoprotein abnormalities, including disorders of lipoprotein function, that might contribute to residual CVD risk in people with T1DM or T2DM. Increasing HDL-C levels using cholesterol ester transfer protein (CETP) inhibitors, was assumed to translate into a clinical benefit. These inhibitors very successfully raised HDL-C in high-risk people but failed to deliver a powerful anti-atherogenic effect [11-14], highlighting that a more comprehensive understanding of HDL biology is needed. HDL particles (HDL-P) exert numerous atheroprotective functions including antioxidant, anti-inflammatory and cholesterol efflux promoting functions [15]. The central role of HDL in reverse cholesterol transport (RCT), is widely considered the key mechanism responsible for HDL-mediated cardioprotection. In the RCT pathway, HDL accepts cholesterol from peripheral cells including lipid-laden foam cells within atherosclerotic lesions and delivers acquired lipid to the liver, for subsequent elimination in the bile and faeces [16, 17]. HDL-cholesterol efflux capacity (HDL-CEC) is driven by the interaction with cellular cholesterol transporters including ATP binding cassette

subfamily A, member 1 (ABCA1), ATP-binding cassette subfamily G, member 1 (ABCG1) and scavenger receptor class B member 1 (SR-B1). In macrophages, small dense lipid-poor HDL-P potently promote efflux via the ABCA1 transporter, while larger HDL-P interact with ABCG1 and SR-B1 transporters [18], and thus different sized particles interact with specific efflux pathways. Measurement of HDL-CEC, as opposed to static HDL-C levels, is now acknowledged as a stronger predictor of CVD risk [19-21], highlighting that HDL quality seems to be more important than its quantity.

The size of HDL-P is also important in accessing their anti-atherogenic properties. Multiple studies have found that higher small HDL-P (S-HDL-P) are most strongly associated with lower CVD risk [22-24]. However, other studies show the opposite and, by extension suggest that large HDL-P (L-HDL-P) are the more protective subclass [25, 26]. These studies vary in design, adjustment for confounders, and methods for HDL subpopulation isolation and quantification, hence, there are conflicting data concerning HDL subpopulations and subsequent prediction of CVD. Working towards resolution of this subclass controversy is ever more important as it is becoming increasingly apparent that measuring the size of lipoprotein particles, and not just the quantity of cholesterol carried on the particles, is another important factor of CVD risk to be considered and elucidated [27].

HDL-P are incredibly complex carrying over 150 lipids [28], 251 proteins [29], and 304 microRNAs [30]. The complexity of the particles makes it challenging to ascertain the contribution of individual proteins to assigned cardioprotective functions of the particles. Furthermore, the composition of the particles differs according to particle size [31], further complicating our ability to understand structure-function relationships. While many studies have analysed the function of HDL *in silo* [19-21], or HDL proteomic composition *in silo* [31, 32], there are few studies that have teased apart the relationship between HDL proteomic composition and HDL function. Furthermore, there are few studies that have investigated the proteomic composition and corresponding efflux capacity of L-HDL-P and S-HDL-P. Given the conflicting data surrounding the association of L-HDL-P and S-HDL-P with CVD, it is pertinent that we get a better understanding of the biology underpinning these different sized particles – collating the particles into one HDL pool may in turn result in loss of critical information relating to different sized particles.

Within this study we sought to understand the impact of both T1DM and T2DM on HDL functionality and HDL protein composition. We investigated how the size and protein composition of HDL affects HDL-CEC in both T1DM and T2DM and respective matched controls.

Study hypothesis:

The overall study hypothesis is that HDL functionality and HDL composition are differentially impacted by T1DM and T2DM and such differences may drive increased CVD risk within these high-risk disease groups.

Objective:

To elucidate how the size and protein composition of HDL impacts HDL function in people with T1DM and people with T2DM and their respective matched controls.

Aims:

The aims of this study were:

1. To compare total CEC, ABCA1-dependent CEC, and ABCA1-independent CEC between people with T1DM, and people with T2DM and their respective age-, sex-, and BMI-matched controls.
2. To investigate the relationship between HDL-P size and HDL-CEC in people with and without T1DM or T2DM.
3. To understand the impact of both T1DM and T2DM on the HDL proteomic composition of L-HDL-P and S-HDL-P and resulting relationship to HDL-CEC.

4.2 Methods

4.2.1 Study design and participants

This was a case-control study designed to compare HDL proteomic composition, HDL size and HDL-CEC between people with T1DM (*n*=81), and people with T2DM (*n*=81) who were age and sex matched, and their respective controls who were age, sex and BMI matched. Two control groups (*n*=81 per control group) were selected as there is a disconnect in BMI between T1DM and T2DM, with people with T2DM generally having a higher BMI. The study design is presented in **Figure 4.1**.

Participants with T1DM or T2DM were contacted either by phone or at the time of their scheduled clinic visit. Inclusion criteria for T1DM were as follows: age 20–65 years; BMI ≥18.5 kg/m^2; duration of diabetes of at least 1 year and HbA1c >6.5%. Inclusion criteria for T2DM were as follows: age 20–65 years; BMI ≥18.5 kg/m^2; duration of diabetes of at least 1 year and HbA1c >6.5%. Exclusion criteria were as follows: pregnant or lactating; and recent illness or any chronic illness likely to influence results. Each participant with T1DM or T2DM was pair-matched with a control volunteer, recruited from the general population. Inclusion criteria were as follows: non-diabetic, age 20–65 years; BMI ≥18.5 kg/m^2; and HbA1c ≤6%. Exclusion criteria were as for participants with T1DM or T2DM. The Diabetes Day Centre in Tallaght University Hospital (TUH), Dublin, recruited all people with diabetes. Control samples were obtained from TUH and the obesity clinic at St. Vincent's University Hospital (SVUH), Dublin.

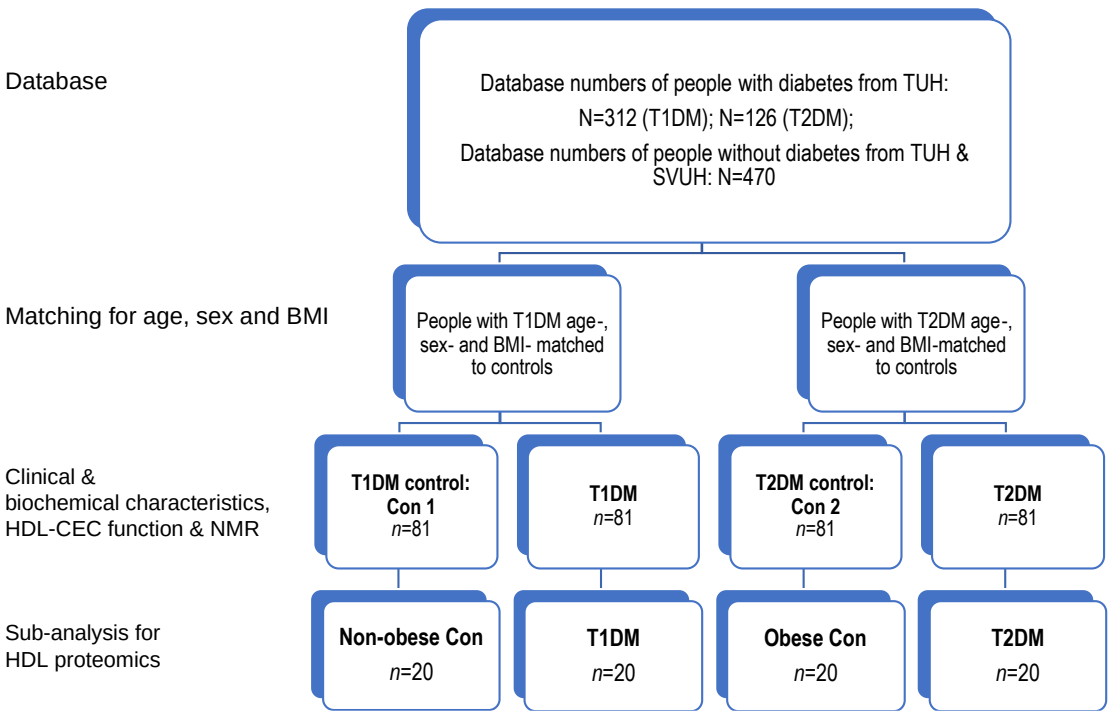


Figure 4.1 *Study design.*

4.2.2 Ethical Approval

All participants gave their written informed consent, which was approved by the Research Ethics Committee of TUH (Dublin, Ireland) & SVUH (Dublin, Ireland).

4.2.3 Study protocol and anthropometric data

All participants were studied after a 12 h fast, having avoided excessive exercise and alcohol for the previous 24 h. Height was measured with a Harpenden stadiometer. Weight was measured in a hospital gown. Completed in TUH and SVUH.

4.2.4 Laboratory methods

Plasma levels of total cholesterol, TG and HDL-C were measured by an enzymatic calorimetric method on the Roche P Module (Roche, Mannheim, Germany). LDL-C was calculated using the Friedewald equation. Additional samples were centrifuged at 3000 rpm for 15 min at 4°C, and serum were stored at −80°C. Completed in TUH and SVUH.

4.2.5 HDL function assays

4.2.5.1 Cholesterol efflux capacity (CEC) [33]

J774.2 murine macrophages, derived from BALB/c mice (European Collection of Authenticated Cell Cultures), were seeded (7×10^5 cells/ml) onto 96-well plates for 24 h, and cultured at 37°C. Cells were subsequently labelled with ^{3}H-cholesterol (1 µCi/ml) for 24 h before being equilibrated for 18 h in DMEM containing 0.2% BSA with or without cAMP (0.3 mmol/l). J774 macrophages do not express ABCA1 basally, and stimulation with cAMP specifically drives ABCA1 protein expression. ApoB-containing lipoproteins were removed from serum by polyethylene glycol (PEG) precipitation, leaving a HDL-enriched supernatant fraction. *Ex vivo* efflux from labelled macrophages to 2.8% HDL-enriched supernatant fraction in minimal essential media (MEM) was measured over 4 h. Control cells were incubated with MEM alone to control for non-specific efflux. ^{3}H-cholesterol levels in the media and remaining cells were calculated by liquid scintillation counting for determination of percentage efflux. Total CEC was determined from cells treated +cAMP. The difference in efflux to HDL from cells stimulated in the presence and absence of cAMP was taken to represent ABCA1-dependent CEC, while ABCA1-independent CEC was calculated from cAMP naive cells.

4.2.6 Nuclear magnetic resonance (NMR) [33]

Lipoprotein particle concentration and sizes were measured in serum samples using 400 MHZ NMR spectroscopy (LipoScience, Raleigh, NC, USA). Each lipoprotein subclass emits a distinctive lipid methyl group NMR signal and the amplitudes of the measured signals are

directly proportional to the number of subclass particles, which in turn provides the estimated mol/l concentration for the three HDL-P subclasses (large 8.9 – 13 nm, medium 8.3 – 8.8 nm, small 7.3 – 8.2 nm) [34]. Weighted-average HDL-P sizes in nanometres were calculated from the subclass levels, and the diameters were assigned to each subclass.

4.2.7 HDL isolation via fast-protein liquid chromatography (FPLC)

Lipoprotein subpopulations (VLDL, LDL, HDL) were isolated from human serum, from a sub cohort of *n*=20 per group, via FPLC. 100 µl of sample, at a serum to PBS containing 1 mM EDTA ratio of 1:1, was injected into the injection valve of the ÄKTA FPLC system (GE Healthcare). The sample processed through two Superose 6 Increase 10/300 GL columns (GE Healthcare, Sweden) placed sequentially, and was eluted into microcentrifuge tubes as 44 separate fractions using a Frac 920 collector (GE Healthcare). Cholesterol content of each freshly eluted fraction was measured by commercial assay (LabAssay™ Cholesterol; Fujifilm) as per manufacturer's instructions. Fractions were stored at -80°C.

4.2.8 HDL Proteomics

Sample preparation for proteomics is described in detail in **Chapter 2, Section 2.7.**

4.2.8.1 Protein isolation

HDL containing fractions 36-38 (large HDL particles, L-HDL-P), and fractions 40-42 (small HDL particles, S-HDL-P) were separately pooled and purified using 30 µl lipid removal agent (LRA) (100mg/ml in 50mM ammonium bicarbonate, Sigma-Aldrich, Ireland) [35] to further enrich for phospholipid-containing HDL-P in the fractions. The LRA pellet was resuspended in 25 µl of 50 mM ammonium bicarbonate containing 2 M Urea.

4.2.8.2 In-solution digestion

Protein samples were in-solution digested with trypsin, (1 vial of trypsin singles at 1 µg size, proteomic grade T7575, Sigma Aldrich, per two samples) at a protein to trypsin ratio of 50:1 (v/v), overnight at 37°C. Peptides were acidified with formic acid (FA), at a final concentration of 1%, to stop trypsin digestion. Tryptic digests were purified using C_{18}ZipTip (Millipore) following the manufactures instructions and resuspended in 3% acetonitrile (ACN) with 0.1% FA, prior to mass spectrometry (MS) analysis.

4.2.8.3 Mass Spectrometry

Peptides were analysed on a quadripole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer fitted with a reversed-phase nano-LC UltiMate 3000 high performance liquid chromatography (HPLC) system (Thermo Scientific).

4.2.8.4 Protein identification

Raw data were processed using the MaxQuant version 1.6.10.43, incorporating the Andromeda search engine. To identify peptides and proteins, MS/MS spectra were matched to the Uniprot *homo sapiens* database (2020_06) containing 76,074 entries. In order to generate label free quantitative (LFQ) ion intensities for protein profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant applying a mass accuracy of at least 20 ppm and a maximum time window of 1 min [36].

4.2.8.5 Proteomic data analysis

Perseus statistical software version 1.6.5.0 was used to analyse the LFQ intensities. Data were log transformed, and t-test comparisons of samples/groups conducted. For visualization using heat maps, missing values were imputed with values from a normal distribution, and the z-score was used to normalise the dataset.

4.2.9 Statistical Analysis

Results in tables are presented as mean ± SD unless otherwise stated. Data in graphs are presented as mean ± SD. Data were tested for normality using the Shapiro–Wilk test, as well as inspected visually using histograms. Statistical significance for categorical variables was calculated using the χ^2 test. For multiple comparisons on normal data, a paired one-way analysis of variance (ANOVA) test was performed with a Bonferroni post-test. The Kruskal-Wallis test with a Dunn's multiple comparison post-test was performed on non-normal data. Correlations were evaluated using the Pearson correlation (normal data) or Spearman test (non-normal data) as appropriate. Multiple regression analysis was used to examine the relationship between several independent variables on one dependent variable.

IBM SPSS Statistics (Version 24.0. Armonk, NY) and GraphPad Prism (Version 9.3.0 San Diego, CA) software for Mac were used for data analysis. Statistical significance is presented

$^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$ for Con1 vs. T1DM;

$^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$, $^{\$\$\$\$}P<0.0001$ for Con1 vs. Con2 (lower versus higher BMI);

$^{⋈}P<0.05$, $^{⋈⋈}P<0.01$, $^{⋈⋈⋈}P<0.001$, $^{⋈⋈⋈⋈}P<0.0001$ for Con1 vs. T2DM;

$^{\S}P<0.05$, $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$, $^{\S\S\S\S}P<0.0001$ for Con2 vs. T2DM; and

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ for T1DM vs. T2DM.

$P>0.05$ was considered non-significant.

4.3 Results

4.3.1 Baseline characteristics of T1DM and T2DM and non-diabetic participants

Clinical and biochemical characteristics of T1DM, T2DM and non-diabetic participants (*n*=81 per group) are shown in **Table *4.1***. The medication use in the four groups is shown in **Table *4.15* in Appendix 4A – Medication Tables.**

All four groups were age- and sex-matched. Total cholesterol and LDL-C were significantly decreased in T1DM compared to its age-, sex- and BMI-matched non-diabetic control (Con1 – lower BMI group). TG were significantly increased in T2DM compared to age-, sex- and BMI-matched non-diabetic control (Con2 – higher BMI group). HbA1c was significantly higher in people with T1DM, and T2DM compared to controls. BMI and TG were significantly increased, while HDL-C was significantly decreased in both Con2 and T2DM compared to age-, and sex-matched Con1. In contrast, BMI and TG were significantly decreased, while HDL-C was significantly increased in T1DM compared to T2DM.

Table 4.1 *Baseline characteristics of T1DM and T2DM and their respective controls.*

Characteristics	Con1 (*n*=81)	T1DM (*n*=81)	Con2 (*n*=81)	T2DM (*n*=81)
Sex (male/ female) (n)	39/42	39/42	39/42	39/42
Age (years)	40.9 ± 9.4	41.2 ± 9.1	41.1 ± 9.2	41.4 ± 9.0
Body mass index (kg/m^2)	25.7 ± 2.5	25.4 ± 2.4	34.6 ± 6.6$^{\$\$\$\$}$	35.0 ± 7.0$^{****, ⋈⋈⋈⋈}$
Smokers (%)	11.1	19.8	13.6	32.1$^{§§,⋈⋈}$
Systolic blood pressure (mm Hg)	126 ± 13	130 ± 17	130 ± 16	128 ± 14
Diastolic blood pressure (mm Hg)	82 ± 10	79 ± 10	83 ± 11	78 ± 10§
Haemoglobin A1c (%)	5.3 ± 0.3	8.2 ± 1.2$^{####}$	5.5 ± 1.2	7.7 ± 2.0$^{§§§§, ⋈⋈⋈⋈}$
Total cholesterol (mmol/l)	4.8 ± 0.8	4.4 ± 0.9$^{#}$	5.0 ± 1.0	4.8 ± 1.8
Triglycerides (mmol/l)	0.9 (0.7-1.5)	0.9 (0.7-1.3)	1.3 (1.0-1.8)$^{\$}$	1.8 (1.3-2.8)$^{****;\ §,\ ⋈⋈⋈⋈}$
LDL cholesterol (mmol/l)	2.8 ± 0.8	2.1 ± 0.9$^{####}$	3.1 ± 0.9	2.6 ± 1.0*
HDL cholesterol (mmol/l)	1.5 ± 0.4	1.7 ± 0.5	1.3 ± 0.4$^{\$\$}$	1.1 ± 0.4$^{****, ⋈⋈⋈⋈}$

Data are displayed as mean ± SD, median (IQR) or %
Statistical significance is presented as
$^{#}P$<0.05, $^{##}P$<0.01, $^{###}P$<0.001, $^{####}P$<0.0001 for Con1 vs. T1DM;
$^{\$}P$<0.05, $^{\$\$}P$<0.01, $^{\$\$\$}P$<0.001, $^{\$\$\$\$}P$<0.0001 for Con1 vs. Con2;
$^{⋈}P$<0.05, $^{⋈⋈}P$<0.01, $^{⋈⋈⋈}P$<0.001, $^{⋈⋈⋈⋈}P$<0.0001 for Con1 vs. T2DM;
$^{§}P$<0.05, $^{§§}P$<0.01, $^{§§§}P$<0.001, $^{§§§§}P$<0.0001 for Con2 vs. T2DM; and
$^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, $^{****}P$<0.0001 for T1DM vs. T2DM.

4.3.2 HDL-CEC function is enhanced in T1DM

A trend towards increased total CEC was observed in people with T1DM (*P*=0.065) relative to their BMI-matched controls with little difference in HDL-CEC evident in T2DM relative to their BMI-matched controls. However, people with T1DM exhibited significantly enhanced total CEC relative to age-, and sex-matched people with T2DM. This effect was largely attributed to a significant increase in ABCA1-dependent efflux in T1DM relative to T2DM, with minimal effects on ABCA1-independent efflux evident. ABCA1-dependent efflux was significantly decreased in T2DM compared to age-, and sex-matched lower BMI controls (Con1).

See **Figure 4.2**.

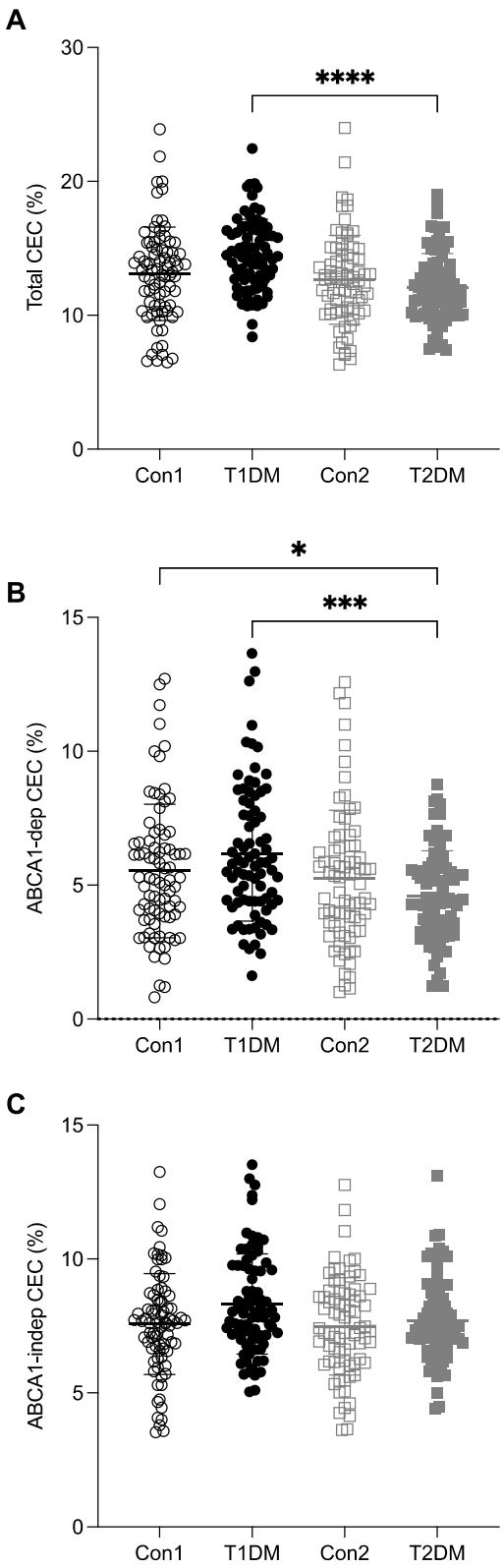


Figure 4.2 *HDL-CEC in participants with T1DM or T2DM and their respective controls.*
HDL-CEC measurements in participants with T1DM or T2DM and their respective controls (*n*=81 per group). J774 macrophages were loaded with ^{3}H-Cholesterol (1µCi/ml) for 24h. Cells were then treated ± cAMP (0.3mM) for 16h and efflux to 2.8% PEG supernatant was monitored over 4h. Dot plots show results for **(A)** total CEC, derived from +cAMP treated cells, **(B)** ABCA1-dependent CEC, calculated as the difference in efflux to PEG-supernatant between cells treated with/ without cAMP**,** and **(C)** ABCA1-independent CEC was calculated from –cAMP treated cells. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

4.3.3 HDL particle (HDL-P) size is significantly modulated in people with T1DM and T2DM

HDL-P size was differentially modulated in people with T1DM vs. T2DM. People with T1DM had significantly increased concentrations of L-HDL-P compared to age- sex-, and BMI-matched controls as well as people with T2DM (**Figure 4.3 A-C**). The total concentration of HDL-P was effectively reduced in T1DM relative to Con1 as well as people with T2DM (**Figure 4.3 D**). The average diameter of HDL-P was also significantly increased in people with T1DM relative to Con1 and T2DM (**Figure 4.3 E**), while cholesterol content on HDL was unchanged relative to Con1, but increased relative to T2DM (**Figure 4.3 F**). By contrast, people with T2DM exhibited a significant increase in the concentration of S-HDL-P relative to people with T1DM (**Figure 4.3 A&C**). Little difference in HDL-P size, concentration and cholesterol content is evident between T2DM and their respective BMI-matched controls indicative that changes in HDL-P metabolism occur early in disease pathogenesis prior to the presentation of T2DM (**Figure 4.3 A-F**).

A sub cohort (*n*=20 per group) underwent FPLC analysis before HDL proteomic composition analysis. Consistent with NMR data, T1DM and T2DM had divergent effects on lipoprotein profiles, an effect that was most pronounced in HDL-containing fractions. Most notably, people with T1DM exhibited more cholesterol on their L-HDL-P (fractions 35-38) compared people with T2DM and relative to age-, sex-, and BMI-matched controls. There were no differences in the cholesterol content on L-HDL-P between T2DM and their age-, sex-, and BMI-matched controls. There were no differences in the cholesterol content of S-HDL-P (fractions 40-42) across groups (**Figure 4.3 G**).

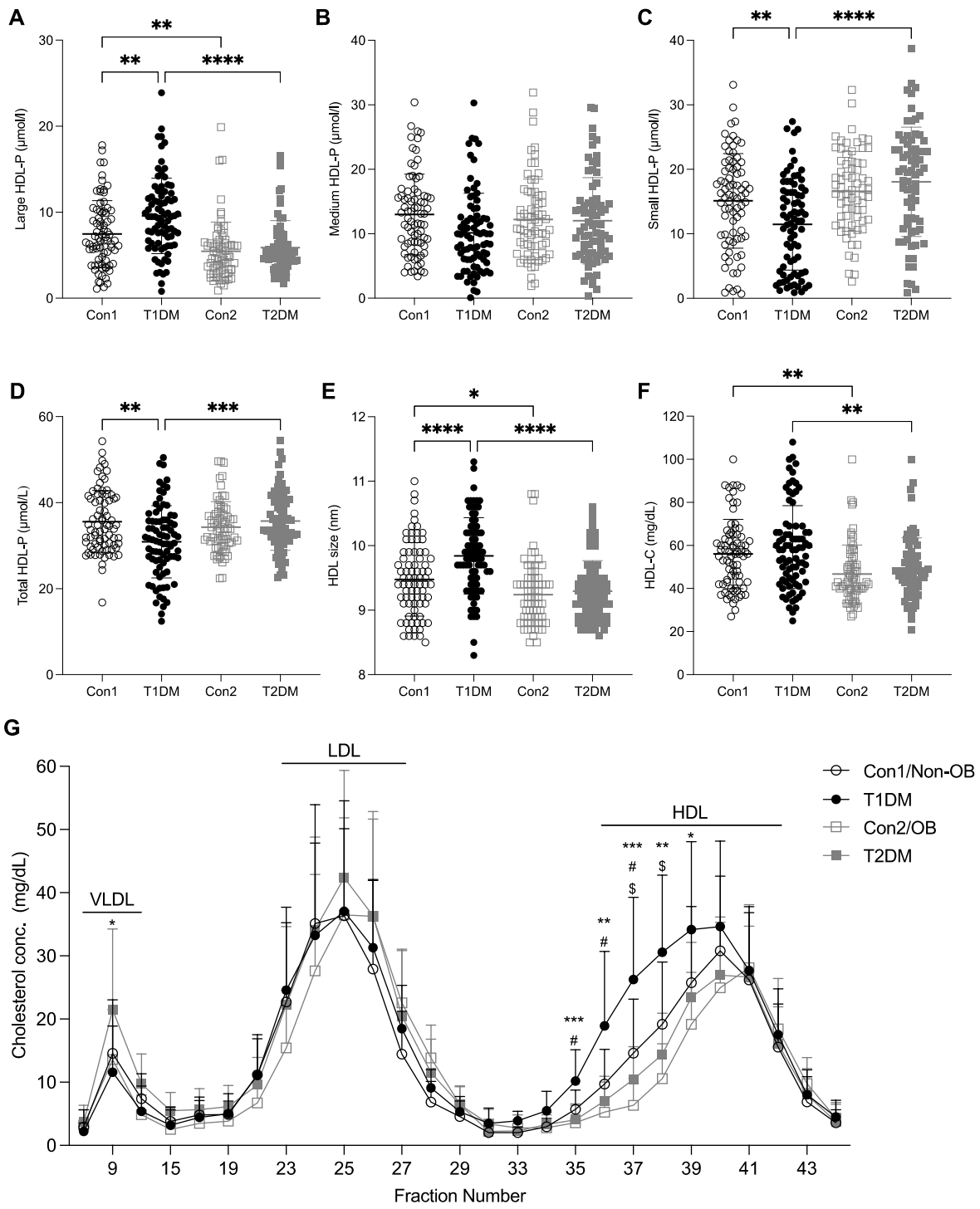


Figure 4.3 *HDL particle size is increased in T1DM compared to T2DM.*
HDL composition analysis in individuals with T1DM or T2DM and their respective controls. 150µL plasma was sent for NMR analysis to LabCorp (LipoScience Inc, NC, USA) (*n*=81 per group). Dot plot shows results for **(A)** Large HDL-P **(B)** Medium HDL-P, **(C)** Small HDL-P, **(D)** Total HDL-P, **(E)** HDL-P diameter, and **(F)** HDL-C. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.
FPLC was performed on serum samples to separate out the VLDL, LDL and HDL fractions. Total cholesterol mass was measured in each fraction in a sub cohort (*n*=20 per group). XY graphs shows result of **(G)** Cholesterol curves. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, and ****P*<0.001, for T1DM vs. T2DM; $^{\#}P$<0.05 for Con1 vs. T1DM; and $^{\$}P$<0.05 for Con1 vs. Con2.

4.3.4 Correlations of HDL-CEC function with HDL concentration, size and cholesterol content

We subsequently investigated the relationship between HDL-CEC, and HDL-P concentration, size and cholesterol content in people with T1DM or T2DM and their respective controls.

Across the total population combined, we identified a significant positive correlation between total CEC and L-HDL-P concentration (r=0.425), medium HDL-P (M-HDL-P) concentration (r=0.152), total HDL-P concentration (r=0.207), HDL-size (r=0.369) and HDL-C levels (r=0.423), with a negative correlation to S-HDL-P concentration (r=−0.148). Similar trends in correlations were also evident for ABCA1-independent CEC, with a particularly strong association to HDL-C levels (r=0.605). By contrast, the association between HDL-P size and ABCA1-dependent CEC was much weaker, with a positive association to L-HDL-P (r=0.194) and HDL size (r=0.223) but no association to M-HDL-P, S-HDL-P or total HDL-P concentrations or HDL-C levels (**Table 4.2**).

In lower BMI controls (Con1), total CEC was significantly positively correlated with L-HDL-P (r=0.288), M-HDL-P (r=0.304) and total HDL-P concentrations (r=0.238), HDL size (r=0.334) and HDL-C levels (r=0.310). There was no correlation between total CEC and the concentration of S-HDL-P in lower BMI controls. Furthermore, there was little relationship evident between HDL-P size and ABCA1-dependent efflux, with much greater correlations evident to ABCA1-independent efflux and HDL-P size parameters in Con1 (**Table 4.2**).

In T1DM, total CEC was significantly positively correlated with L-HDL-P (r=0.313) and M-HDL-P concentrations (r=0.224), and HDL-C levels (r=0.310). ABCA1-independent efflux similarly positively correlated with L-HDL-P (r=0.296) and M-HDL-P concentrations (r=0.662), HDL-C levels (r=0.605), and also total HDL-P concentration (r=0.695). In contrast, ABCA1-dependent CEC was significantly negatively correlated M-HDL-P (r=−0.255), S-HDL-P (r=−0.277) and total HDL-P concentrations (r=−0.365) (**Table 4.2**).

Within the higher BMI control group (Con2), total CEC was significantly positively correlated with L-HDL-P (r=0.596), total HDL-P concentration (r=0.434), HDL size (r=0.466) and HDL-C levels (r=0.569) with no correlation to M-HDL-P and S-HDL-P. In Con2, ABCA1-independent CEC followed similar trends as total CEC in relation to HDL-P size parameters with a particularly strong association to HDL-C (r=0.623) and further negative association with S-HDL-P concentration (r=−0.240). Weak associations to ABCA1-dependent efflux were again evident with a significant positive association to L-HDL-P concentration (r=0.355) and HDL-C (r=0.309). Interestingly, total HDL-P concentration was significantly and positively associated with ABCA1-dependent CEC in Con2 (r=0.273) and T2DM (r=0.299), with an opposing association in T1DM (r=−0.365) and no association in lower BMI controls (r=−0.05) (**Table 4.2**).

In T2DM, total CEC was significantly positively correlated with L-HDL-P (r=0.244), S-HDL-P (r=0.287), total HDL-P concentration (r=0.560), and HDL-C levels (r=0.435). ABCA1-independent CEC positively correlated with L-HDL-P concentrations (r=0.504) and HDL-C levels (r=0.666). In contrast to the other groups, ABCA1-dependent CEC positively correlated with S-HDL-P concentrations in people with T2DM (r=0.412) (**Table 4.2**).

Table 4.2 *Correlations of HDL-CEC parameters with HDL concentration, size and cholesterol content.*

Characteristics	All (*n*=324)		Con1 – Lower BMI (*n*=81)		T1DM (*n*=81)		Con2 – Higher BMI (*n*=81)		T2DM (*n*=81)	
	r	*P* value	r	*P* value	r	*P* value	r	*P* value	r	*P* value
Total CEC										
Large HDL-P conc. (µmol/l)	0.425	<0.0001****	0.288	0.011*	0.313	0.004**	0.596	<0.0001****	0.244	0.037*
Medium HDL-P conc. (µmol/l)	0.152	0.008**	0.304	0.007**	0.224	0.044*	0.166	0.170	0.083	0.486
Small HDL-P conc. (µmol/l)	−0.148	0.0096**	−0.177	0.121	−0.212	0.057	−0.076	0.534	0.287	0.014*
Total HDL-P conc.(µmol/l)	0.207	0.0003***	0.238	0.036*	0.144	0.201	0.434	0.0002***	0.560	<0.0001****
HDL size (nm)	0.369	<0.0001****	0.334	0.003**	0.211	0.060	0.466	<0.0001****	0.090	0.448
HDL-C (mg/dL)	0.423	<0.0001****	0.310	0.006**	0.310	0.005**	0.569	<0.0001****	0.435	0.0001***
ABCA1-dependent CEC										
Large HDL-P conc. (µmol/l)	0.194	0.0007***	0.029	0.798	0.119	0.290	0.355	0.0026**	−0.121	0.307
Medium HDL-P conc. (µmol/l)	−0.066	0.248	0.205	0.071	−0.255	0.02*	0.004	0.975	−0.172	0.145
Small HDL-P conc. (µmol/l)	−0.092	0.112	−0.108	0.335	−0.277	0.012*	0.065	0.593	0.412	0.0003***
Total HDL-P conc.(µmol/l)	−0.05	0.407	0.083	0.468	−0.365	0.0008***	0.273	0.022*	0.299	0.010*
HDL size (nm)	0.223	0.0001***	0.155	0.175	0.184	0.100	0.269	0.025*	−0.148	0.210
HDL-C (mg/dL)	0.097	0.091	0.031	0.789	−0.123	0.274	0.309	0.009**	−0.006	0.962
ABCA1-independent CEC										
Large HDL-P conc. (µmol/l)	0.476	<0.0001****	0.498	<0.0001****	0.296	0.0098**	0.605	<0.0001****	0.504	<0.0001****
Medium HDL-P conc. (µmol/l)	0.352	<0.0001****	0.291	0.0097**	0.662	<0.0001****	0.310	0.009**	0.315	0.0067**
Small HDL-P conc. (µmol/l)	−0.134	0.02*	−0.185	0.105	0.071	0.531	−0.240	0.045*	−0.012	0.869
Total HDL-P conc.(µmol/l)	0.425	<0.0001****	0.332	0.003**	0.695	<0.0001****	0.420	0.0003***	0.518	<0.0001****
HDL size (nm)	0.341	<0.0001****	0.415	0.0002***	0.054	0.635	0.487	<0.0001****	0.300	0.001**
HDL-C (mg/dL)	0.605	<0.0001****	0.539	<0.0001****	0.605	<0.0001****	0.623	<0.0001****	0.666	<0.0001****

Correlation coefficient (r)

+1 Perfect
+0.800 - 0.999 V. strong
+0.600 - 0.799 Strong
+0.400 - 0.599 Moderate
+0.200 - 0.399 Weak
+0.000 - 0.199 V. Weak
Non-sig
−0.000 - −0.199 V. Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 V. strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.5 Associations of clinical, biochemical and medication variables with HDL-CEC parameters

Multiple regression analysis was used to assess the influence of selected variables on HDL-CEC when combined into models i.e. to assess for confounding effects. Dummy variables were used to investigate the effects of both T1DM and T2DM on HDL-CEC parameters within the models.

Model 1 incorporates sex, smoking status, age, BMI, total cholesterol, TG, HDL-C, total HDL-P concentration, HDL size, T1DM status and T2DM status, while Model 2 incorporates all the variables from Model 1 as well as medications including ACE-I/ARB, statin, metformin, gliclazide, GLP-1, and DPP4.

Total efflux was significantly positively associated with HDL-C, total HDL-P concentration and T1DM status and negatively associated with smoking in Model 1. Taking into account the medication profiles, all the variables from Model 1 retained their significance. Additionally, total efflux was significantly positively associated with TG and negatively associated with T2DM status. Both models accounted for only 27.5% of the observed variation (**Table 4.3**).

ABCA1-dependent CEC was significantly positively associated with TG and negatively associated with smoking and T2DM status in Model 1. This model accounted for 11.1% of the variation observed. Taking into account the medication profiles, all the variables from Model 1 retained their significance and no other variable was significant. Furthermore, Model 2 accounted for less of the observed variation (10.6%) compared to Model 1 (**Table 4.4**).

ABCA1-independent CEC was significantly positively associated with total HDL-P concentration, HDL size and T1DM status in Model 1. This model accounted for 37.4% of the variation observed. Taking into account the medication profiles, all the variables from Model 1 retained their significance and no other variable was significant. Furthermore, Model 2 accounted for less of the observed variation (36.8%) compared to Model 1 (**Table 4.5**).

Table 4.3 *Beta coefficients for the association of clinical, biochemical and medication variables with total efflux.*

Model 1	β coefficient	95% CI		Model 2	β coefficient	95% CI	
		Lower Bound	Upper Bound			Lower Bound	Upper Bound
(Constant)	-1.528	-10.910	7.855	(Constant)	-4.284	-14.278	5.709
Sex (1 man, 2 woman)	0.671	-0.220	1.562	Sex (1 man, 2 woman)	0.800	-0.112	1.712
Smoker (1 no, 2 yes)	−1.093*	-1.967	-0.219	Smoker (1 no, 2 yes)	−1.146*	-2.036	-0.256
Age (years)	-0.004	-0.048	0.040	Age (years)	-0.014	-0.061	0.032
BMI (kg/m^2)	0.018	-0.051	0.087	BMI (kg/m^2)	0.013	-0.059	0.086
Total cholesterol (mmol/l)	0.055	-0.317	0.427	Total cholesterol (mmol/l)	0.088	-0.291	0.467
Triglycerides (mmol/l)	0.477	-0.026	0.980	Triglycerides (mmol/l)	0.509*	0.002	1.015
HDL cholesterol (mmol/l)	1.714*	0.324	3.103	HDL cholesterol (mmol/l)	1.733*	0.340	3.127
Total HDL-P conc.(µmol/l)	0.082*	0.020	0.145	Total HDL-P conc.(µmol/l)	0.075*	0.012	0.139
HDL size (nm)	0.858	-0.054	1.771	HDL size (nm)	0.906	-0.014	1.827
T1DM status	1.396**	0.431	2.361	ACE-I/ARB (1 no, 2 yes)	-0.713	-2.118	0.692
T2DM status	-0.755	-1.777	0.267	Statin (1 no, 2 yes)	0.972	-0.452	2.395
				Metformin (1 no, 2 yes)	1.300	-0.386	2.985
				Diamicron (1 no, 2 yes)	-0.063	-1.954	1.827
				GLP-1 (1 no, 2 yes)	0.106	-1.861	2.072
				DPP4 (1 no, 2 yes)	1.092	-1.134	3.319
				T1DM status	1.291*	0.227	2.356
				T2DM status	−1.921*	-3.521	-0.321
Adjusted R^2=0.275				Adjusted R^2=0.275			

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 4.4 *Beta coefficients for the association of clinical, biochemical and medication variables with ABCA1-dependent CEC.*

Model 1	β coefficient	95% CI		Model 2	β coefficient	95% CI	
		Lower Bound	Upper Bound			Lower Bound	Upper Bound
(Constant)	3.214	-4.942	11.370	(Constant)	1.063	-7.650	9.777
Sex (1 man, 2 woman)	0.635	-0.139	1.410	Sex (1 man, 2 woman)	0.739	-0.056	1.534
Smoker (1 no, 2 yes)	−0.867*	-1.627	-0.108	Smoker (1 no, 2 yes)	−0.917*	-1.693	-0.141
Age (years)	-0.011	-0.049	0.028	Age (years)	-0.016	-0.057	0.024
BMI (kg/m^2)	0.005	-0.055	0.065	BMI (kg/m^2)	-0.002	-0.065	0.061
Total cholesterol (mmol/l)	-0.003	-0.326	0.320	Total cholesterol (mmol/l)	0.019	-0.312	0.349
Triglycerides (mmol/l)	0.494*	0.057	0.932	Triglycerides (mmol/l)	0.517*	0.075	0.959
HDL cholesterol (mmol/l)	1.031	-0.177	2.239	HDL cholesterol (mmol/l)	1.059	-0.156	2.274
Total HDL-P conc.(µmol/l)	-0.024	-0.079	0.030	Total HDL-P conc.(µmol/l)	-0.030	-0.085	0.025
HDL size (nm)	0.133	-0.661	0.926	HDL size (nm)	0.148	-0.654	0.950
T1DM status	0.546	-0.293	1.385	ACE-I/ARB (1 no, 2 yes)	-0.356	-1.581	0.869
T2DM status	−1.01*	-1.899	-0.122	Statin (1 no, 2 yes)	0.480	-0.761	1.721
				Metformin (1 no, 2 yes)	1.094	-0.376	2.564
				Diamicron (1 no, 2 yes)	-0.516	-2.165	1.132
				GLP-1 (1 no, 2 yes)	0.513	-1.202	2.227
				DPP4 (1 no, 2 yes)	1.106	-0.835	3.047
				T1DM status	0.473	-0.455	1.401
				T2DM status	−1.951**	-3.346	-0.556
Adjusted R^2=0.111				Adjusted R^2=0.106			

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 4.5 *Beta coefficients for the association of clinical, biochemical and medication variables with ABCA1-independent CEC.*

Model 1	β coefficient	95% CI		Model 2	β coefficient	95% CI	
		Lower Bound	Upper Bound			Lower Bound	Upper Bound
(Constant)	-4.743	-9.653	0.166	(Constant)	-5.346	-10.602	-0.089
Sex (1 man, 2 woman)	0.035	-0.432	0.501	Sex (1 man, 2 woman)	0.06	-0.42	0.54
Smoker (1 no, 2 yes)	-0.225	-0.683	0.232	Smoker (1 no, 2 yes)	-0.228	-0.697	0.24
Age (years)	0.007	-0.016	0.03	Age (years)	0.002	-0.023	0.027
BMI (kg/m^2)	0.013	-0.023	0.05	BMI (kg/m^2)	0.016	-0.023	0.054
Total cholesterol (mmol/l)	0.058	-0.136	0.253	Total cholesterol (mmol/l)	0.069	-0.13	0.269
Triglycerides (mmol/l)	-0.018	-0.281	0.246	Triglycerides (mmol/l)	-0.009	-0.275	0.258
HDL cholesterol (mmol/l)	0.684	-0.043	1.411	HDL cholesterol (mmol/l)	0.676	-0.057	1.409
Total HDL-P conc.(µmol/l)	0.107****	0.074	0.139	Total HDL-P conc.(µmol/l)	0.105****	0.072	0.138
HDL size (nm)	0.725**	0.248	1.203	HDL size (nm)	0.758**	0.274	1.242
T1DM status	0.85**	0.345	1.355	ACE-I/ARB (1 no, 2 yes)	-0.357	-1.096	0.382
T2DM status	0.255	-0.28	0.789	Statin (1 no, 2 yes)	0.491	-0.258	1.24
				Metformin (1 no, 2 yes)	0.205	-0.681	1.092
				Diamicron (1 no, 2 yes)	0.452	-0.542	1.447
				GLP-1 (1 no, 2 yes)	-0.409	-1.443	0.626
				DPP4 (1 no, 2 yes)	-0.015	-1.186	1.156
				T1DM status	0.818**	0.258	1.378
				T2DM status	0.03	-0.811	0.872
Adjusted R^2=0.374				Adjusted R^2=0.368			

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.6 Baseline characteristics of sub cohort for HDL proteomics

A sub cohort of *n*=20 per group was brought forward for HDL proteomic composition analysis to further probe the relationship between HDL protein composition and HDL-CEC.

Participants selected with T1DM and their respective controls all had a BMI ≤29.9 kg/m^2 (non-obese), while participants selected with T2DM and their respective controls all had a BMI ≥30 kg/m^2 (obesity).

Clinical and biochemical characteristics of the sub-cohort of T1DM, T2DM and their respective age-, sex-, and BMI-matched non-diabetic participants are shown in **Table 4.6**. The medication use in the four groups is shown in **Table *4.16*** in **Appendix 4A – Medication Tables.**

Table 4.6 *Baseline characteristics of a sub-cohort of T1DM and T2DM and their respective controls.*

Characteristic	Non-OB (Con1) (*n*=20)	T1DM (*n*=20)	OB (Con2) (*n*=20)	T2DM (*n*=20)
Clinical/ biochemical				
Sex (male/ female) (n)	10/10	10/10	10/10	10/10
Age (years)	43.2 ± 7.0	43.1 ± 6.6	43.1 ± 7.4	43.4 ± 6.2
Body mass index (kg/m^2)	25.1 ± 2.8	25.0 ± 2.7	35.1 ± 3.0$^{\$\$\$\$}$	35.1 ± 3.8$^{****,⋈⋈⋈⋈}$
Smokers (%)	15	35	15	15
Systolic blood pressure (mm Hg)	130 ± 8	128 ± 11	134 ± 15	128 ± 16
Diastolic blood pressure (mm Hg)	82 ± 9	80 ± 4	86 ± 13	77 ± 9
Haemoglobin A1c (%)	5.3 ± 0.2	8.1 ± 1.1$^{####}$	5.4 ± 0.3	7.5 ± 1.6$^{§§,⋈⋈}$
Total cholesterol (mmol/l)	5.1 ± 0.9	4.5 ±0.7	5.6 ± 1.1	4.3 ± 1.3§
Triglycerides (mmol/l)	1.0 (0.8-1.3)	1.1 (0.8-1.3)	1.4 (1.2-2.6)	2.3 (1.0-3.5)$^{*,⋈}$
LDL cholesterol (mmol/l)	3.0 ± 0.8	2.1 ±0.8$^{#}$	3.5 ± 1.0	2.0 ± 0.8$^{§§,⋈⋈}$
HDL cholesterol (mmol/l)	1.6 ± 0.4	1.8 ± 0.5	1.2 ± 0.4	1.0 ± 0.3$^{***,⋈⋈}$
HDL functionality				
Total CEC (%efflux/4hr)	12.58 ± 3.18	14.38 ± 2.5	12.39 ± 4.19	12.49 ± 2.61
ABCA1-dependent CEC (%efflux/4hr)	5.28 ± 2.7	5.59 ± 2.12	5.08 ±2.83	5.25 ± 2.14
ABCA1-independent CEC (%efflux/4hr)	7.3 ± 1.90	8.79 ± 1.98	7.62 ± 2.12	7.49 ± 1.35

Data are displayed as mean ± SD, median (IQR) or %
Statistical significance is presented as
$^{#}P$<0.05, $^{##}P$<0.01, $^{###}P$<0.001, $^{####}P$<0.0001 for Con1 vs. T1DM;
$^{\$}P$<0.05, $^{\$\$}P$<0.01, $^{\$\$\$}P$<0.001, $^{\$\$\$\$}P$<0.0001 for Con1 vs. Con2;
$^{⋈}P$<0.05, $^{⋈⋈}P$<0.01, $^{⋈⋈⋈}P$<0.001, $^{⋈⋈⋈⋈}P$<0.0001 for Con1 vs. T2DM;
$^{§}P$<0.05, $^{§§}P$<0.01, $^{§§§}P$<0.001, $^{§§§§}P$<0.0001 for Con2 vs. T2DM; and
$^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, $^{****}P$<0.0001 for T1DM vs. T2DM.
Non-OB = Non-obesity, T1DM = type 1 diabetes mellitus; OB = obesity; T2DM = type 2 diabetes mellitus.

4.3.7 The HDL proteomic composition of large HDL-P and small HDL-P is profoundly different

Given the strong association between L-HDL-P concentration and measures of HDL-CEC and weaker associations between S-HDL-P and HDL-CEC, coupled with opposing effects of T1DM and T2DM on HDL-P size, we sought to characterize the HDL proteomic composition of L-HDL-P and S-HDL-P separately to gain greater insight into structure/function relationships. It is noteworthy that the composition of large vs. small HDL-P are profoundly different, further justifying the approach to analyse the different sub-populations in people with and without T1DM or T2DM. We initially pooled all subjects together for this analysis regardless of metabolic disease status (*n*=80 in total per group).

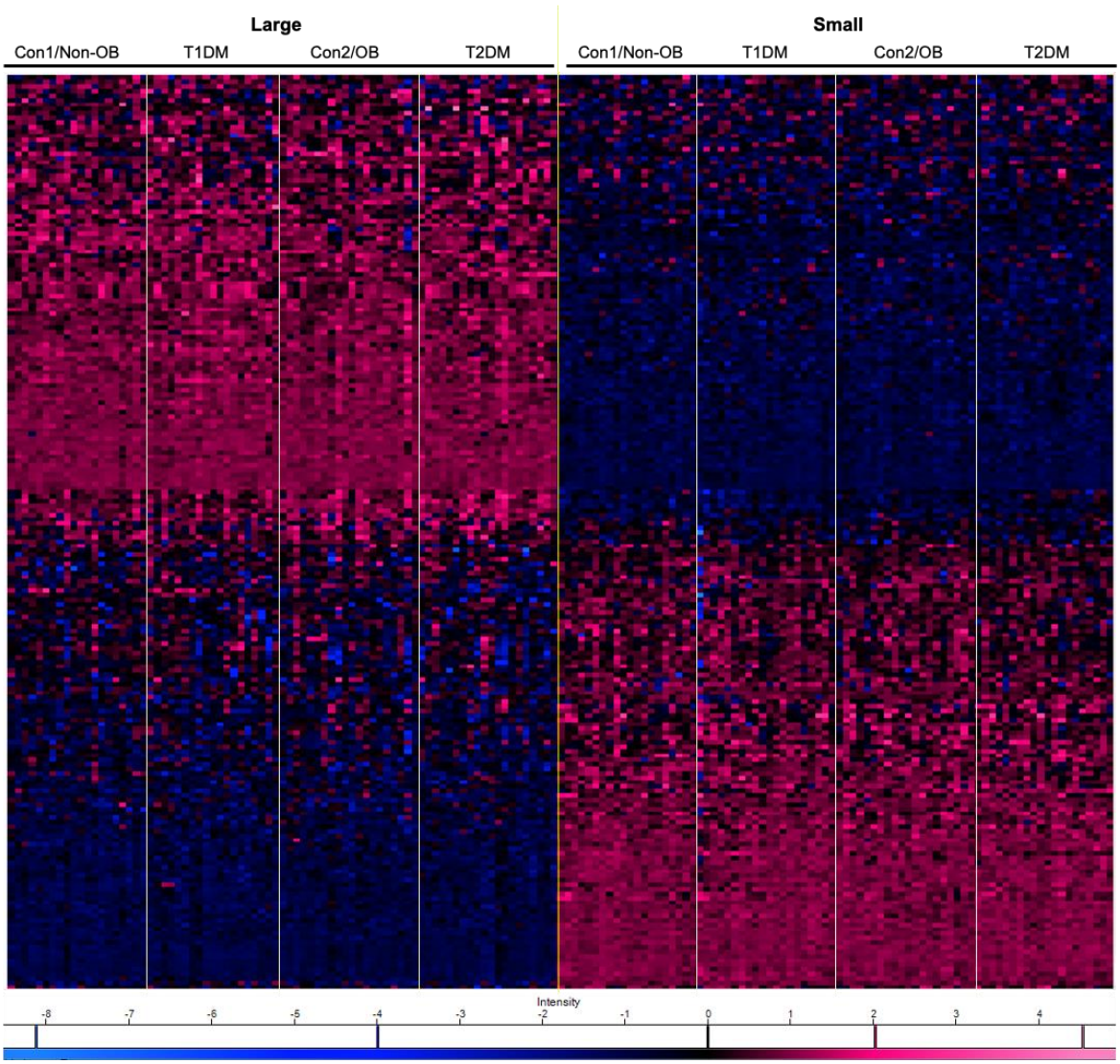
590 valid proteins were detected on L-HDL-P and S-HDL-P after MS. 224 proteins were identified on L-HDL-P and S-HDL-P from all four groups after filtering for 70% valid proteins in at least one group. Of these, 205 were significantly different between L-HDL-P and S-HDL-P (**Figure 4.4 A**). 194 proteins reached the statistical threshold of the false discovery rate (FDR) of 5% ($-Log_{10}$=3 in volcano plot), which adjusts for multiple comparisons in large datasets. Of these, 101 were enriched and 93 were depleted on L-HDL-P (**Figure 4.4 D**). Inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1) and H2 (ITIH2) were two of the most enriched proteins on L-HDL-P, while hemopexin (HPX) and alpha-1B-glycoprotein (A1BG) were the most enriched on S-HDL-P.

Delving into proteins of interest, we found that apolipoproteins (Apo)A-I and ApoA-II were enriched on S-HDL-P, while other apolipoproteins including ApoA-IV, ApoC-II, ApoC-IV, ApoD, ApoE and ApoM were enriched on L-HDL-P. ApoC-III was enriched on L-HDL-P, however, its significance was lost when the FDR was applied (**Figure 4.4 B**).

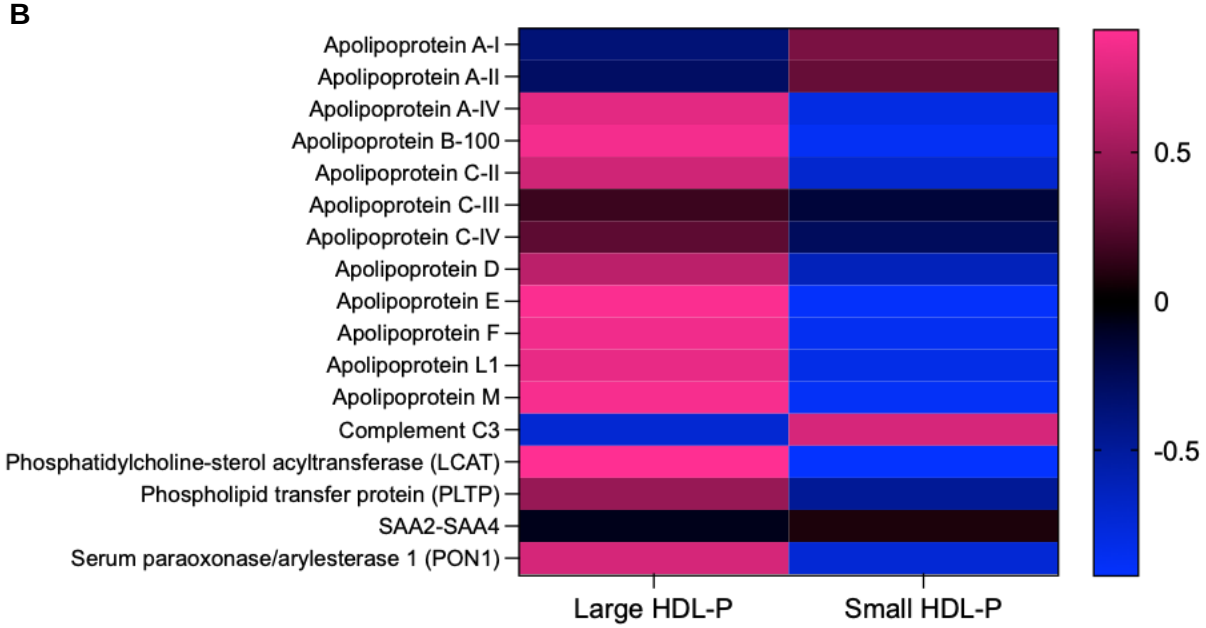
The HDL remodelling proteins phosphatidylcholine-sterol acyltransferase/ lecithin-cholesterol acyltransferase (LCAT) and phospholipid transfer protein (PLTP), and the antioxidant, serum paraoxonase/arylesterase 1 (PON1), were enriched on L-HDL-P. There was no difference in the pro-inflammatory protein serum amyloid A (SAA) 2-4 between L-HDL-P and S-HDL-P, while complement C3, was enriched on S-HDL-P (**Figure 4.4 B**). There was also enrichment of the immunoglobulins (Ig) on S-HDL-P relative to L-HDL-P (**Figure 4.4 C**).

We therefore sought to characterise the HDL proteomic composition of L-HDL-P and S-HDL-P in people with and without T1DM or T2DM.

A


Large
Con1/Non-OB
T1DM
Con2/OB
T2DM
Small
Con1/Non-OB
T1DM
Con2/OB
T2DM
Intensity
-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4

B


Apolipoprotein A-I
Apolipoprotein A-II
Apolipoprotein A-IV
Apolipoprotein B-100
Apolipoprotein C-II
Apolipoprotein C-III
Apolipoprotein C-IV
Apolipoprotein D
Apolipoprotein E
Apolipoprotein F
Apolipoprotein L1
Apolipoprotein M
Complement C3
Phosphatidylcholine-sterol acyltransferase (LCAT)
Phospholipid transfer protein (PLTP)
SAA2-SAA4
Serum paraoxonase/arylesterase 1 (PON1)
Large HDL-P
Small HDL-P
0.5
0
-0.5

C

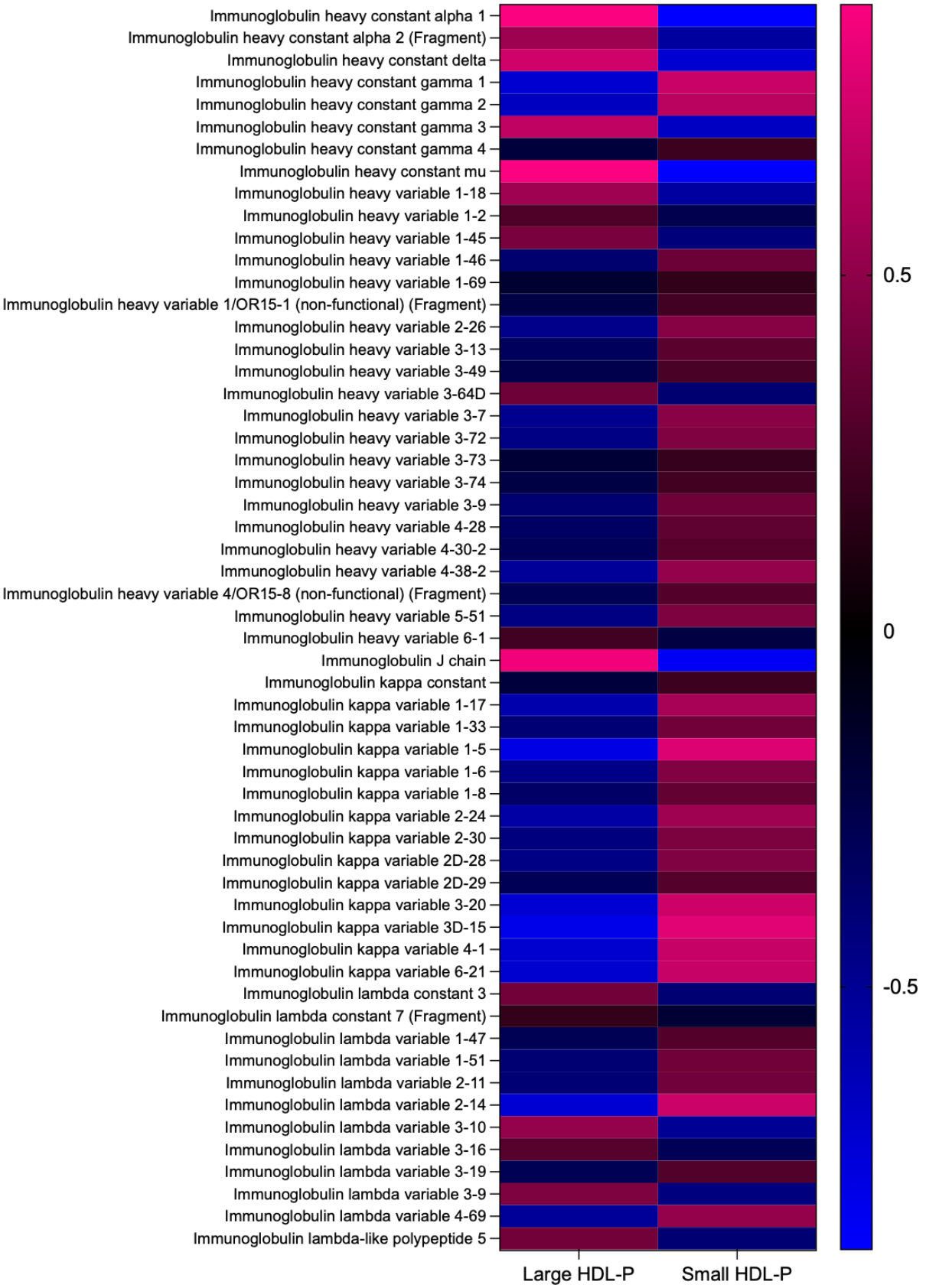

Immunoglobulin heavy constant alpha 1
Immunoglobulin heavy constant alpha 2 (Fragment)
Immunoglobulin heavy constant delta
Immunoglobulin heavy constant gamma 1
Immunoglobulin heavy constant gamma 2
Immunoglobulin heavy constant gamma 3
Immunoglobulin heavy constant gamma 4
Immunoglobulin heavy constant mu
Immunoglobulin heavy variable 1-18
Immunoglobulin heavy variable 1-2
Immunoglobulin heavy variable 1-45
Immunoglobulin heavy variable 1-46
Immunoglobulin heavy variable 1-69
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)
Immunoglobulin heavy variable 2-26
Immunoglobulin heavy variable 3-13
Immunoglobulin heavy variable 3-49
Immunoglobulin heavy variable 3-64D
Immunoglobulin heavy variable 3-7
Immunoglobulin heavy variable 3-72
Immunoglobulin heavy variable 3-73
Immunoglobulin heavy variable 3-74
Immunoglobulin heavy variable 3-9
Immunoglobulin heavy variable 4-28
Immunoglobulin heavy variable 4-30-2
Immunoglobulin heavy variable 4-38-2
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)
Immunoglobulin heavy variable 5-51
Immunoglobulin heavy variable 6-1
Immunoglobulin J chain
Immunoglobulin kappa constant
Immunoglobulin kappa variable 1-17
Immunoglobulin kappa variable 1-33
Immunoglobulin kappa variable 1-5
Immunoglobulin kappa variable 1-6
Immunoglobulin kappa variable 1-8
Immunoglobulin kappa variable 2-24
Immunoglobulin kappa variable 2-30
Immunoglobulin kappa variable 2D-28
Immunoglobulin kappa variable 2D-29
Immunoglobulin kappa variable 3-20
Immunoglobulin kappa variable 3D-15
Immunoglobulin kappa variable 4-1
Immunoglobulin kappa variable 6-21
Immunoglobulin lambda constant 3
Immunoglobulin lambda constant 7 (Fragment)
Immunoglobulin lambda variable 1-47
Immunoglobulin lambda variable 1-51
Immunoglobulin lambda variable 2-11
Immunoglobulin lambda variable 2-14
Immunoglobulin lambda variable 3-10
Immunoglobulin lambda variable 3-16
Immunoglobulin lambda variable 3-19
Immunoglobulin lambda variable 3-9
Immunoglobulin lambda variable 4-69
Immunoglobulin lambda-like polypeptide 5
0.5
0
-0.5
Large HDL-P
Small HDL-P

Figure 4.4 *Significantly changed proteins between large HDL-P and small HDL-P across all groups combined.*

HDL was isolated via FPLC from people with and without T1DM or T2DM (n=20 per group; n=80 in total). Differences in HDL protein levels between L-HDL-P (fractions 36-38) and S-HDL-P (fractions 40-42) when all groups were combined were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (P<0.05) changed proteins and presented in a heatmap**. (B)** Mean values of proteins of interest on L-HDL-P and S-HDL-P. **(C)** Mean values of immunoglobulins on L-HDL-P and S-HDL-P. **(D)** Volcano plot showing the differences in protein abundance detected between L-HDL-P and S-HDL-P across all groups. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a P value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down on L-HDL-P vs. S-HDL-P.

4.3.8 Modulation of the proteomic composition of L-HDL-P

We identified changes in the pattern of HDL-associated proteins on L-HDL-P between people with T1DM or T2DM and their respective age, sex and BMI-matched controls (*n*=20 per group). 590 valid proteins were detected on L-HDL-P after MS.

4.3.8.1 Modulation of the proteomic composition of L-HDL-P in T1DM

211 proteins were identified on L-HDL-P from both non-obese controls (non-OB) and T1DM after filtering for 14 valid proteins (70%) in at least one group. 15 significantly different proteins on L-HDL-P were identified between non-OB and T1DM. Of these, five HDL-associated proteins were significantly decreased in T1DM compared to non-OB controls, including the pro-inflammatory protein complement C3 (C3); and antithrombin-III (SERPINC1) and prothrombin (F2), which have functions in blood coagulation. By contrast, ten HDL-associated proteins were significantly increased in T1DM compared to non-OB controls, including complement component C8 gamma chain (C8G), and C4b-binding protein alpha chain (C4BPA), which are involved in the complement system; extracellular superoxide dismutase [Cu-Zn] (SOD3), which is involved in mitigating against oxidative stress; beta-2-glycoprotein 1 (APOH), which is involved in blood coagulation; the acute-phase response protein SAA2-SAA4; and also ApoM which is involved in cholesterol and lipoprotein metabolism (**Table *4.7*** & **Figure 4.10 A&B in Appendix 4D**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}$=3 in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 4.10 B in Appendix 4D**).

4.3.8.2 Modulation of the proteomic composition of L-HDL-P in T2DM

212 proteins were identified on L-HDL-P from both OB controls and T2DM after filtering for 14 valid proteins (70%) in at least one group. 16 significantly different proteins on L-HDL-P were identified between OB controls and T2DM. Of these, seven HDL-associated proteins were significantly decreased in T2DM compared to its age-, sex-, and BMI-matched control, including the most abundant HDL protein, ApoA-I; alpha-1-antitrypsin (SERPINA1), which is involved in blood coagulation; gelsolin (GSN); A-kinase anchor protein 9 (AKAP9), and three Igs.

By contrast, nine HDL-associated proteins were significantly increased in T2DM compared to OB controls including complement component C7 (C7); kininogen-1 (KNG1), Ig heavy variable 2-70D (IGHV2-70D) and Ig heavy variable 3-73 IGHV3-73) which are involved in the immune response; coagulation factor V (F5) and plasma kallikrein (KLKB1) which are involved in blood coagulation; and also the antioxidant protein ApoA-IV (**Table *4.7*** & **Figure 4.11 A&B in Appendix 4D**).

KLKB1 and ApoA-IV were the only two proteins to reach the statistical threshold of the FDR of 5% ($-Log_{10}$=3 in volcano plot), which adjusts for multiple comparisons in large datasets (**Table *4.7*** & **Figure 4.11 B in Appendix 4D**).

4.3.8.3 Modulation of the proteomic composition of L-HDL-P in control subjects with obesity relative to age- and sex-matched people without obesity

214 proteins were identified on L-HDL-P from control groups with and without obesity after filtering for 14 valid proteins (70%) in at least one group. 32 significantly different proteins on L-HDL-P were identified between people with and without obesity. Of these, 20 HDL-associated proteins were significantly increased in obesity compared to the non-obesity group including complement proteins, complement C3 (C3), C3/C5 convertase (C3/C5), complement C5 (C5), and C4b-binding protein alpha chain (C4BPA); fibrinogen alpha chain (FGA), heparin cofactor 2 (SERPIND1) and vitamin K-dependent protein Z (PROS1), which are involved in blood coagulation; clusterin (CLU), and Ig heavy variable 2-70D, which are involved in the immune response; alpha-1-acid glycoprotein 2 (ORMA), and serum amyloid P-component (APCS), which are involved in the acute-phase response; and ApoL1. By contrast, 12 HDL-associated proteins were significantly reduced in obesity compared to the non-obesity group, nine of which are involved in the immune response as well as the antioxidant protein PON1 and the apolipoproteins ApoA-I and ApoD (**Table *4.7*** & **Figure 4.12 A&B in Appendix 4D**).
No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}$=3 in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 4.12 B in Appendix 4D**).

4.3.8.4 Modulation of the proteome composition of L-HDL-P in the combined high BMI (OB&T2DM) group relative to non-obese group

We subsequently combined obesity and T2DM groups together to compare all subjects with high BMI within this study to non-obese counterparts.
212 proteins were identified on L-HDL-P from both non-obese and combined obesity group after filtering for 70% valid proteins in at least one group. 33 significantly different proteins on L-HDL-P were identified between non-obese and combined obesity group. Of these, 20 HDL-associated proteins were significantly increased in the combined obesity group compared to non-obese group, including the complement proteins, complement factor 1 (CF1) complement component C8 gamma chain (C8G)**,** C4b-binding protein alpha chain (C4BPA), and complement C5 (C5)**;** plasma kallikrein (KLKB1)**,** heparin cofactor 2 (SERPIND1), and fibrinogen alpha chain (FGA), which are involved in blood coagulation; alpha-1-acid glycoprotein 2 (AGP2), and serum amyloid P-component (APCS), which are involved in the acute-phase response; and ApoL1. By contrast, 13 HDL-associated proteins were significantly

reduced in the combined obesity group compared to non-obese group, seven of which are involved in the immune response, and others including the antioxidant proteins PON1 and PON3, and the apolipoproteins ApoF, ApoD ApoA-I and ApoM (**Table *4.7*** & **Figure 4.13 A&B in Appendix 4D**). These findings highlight that changes in the HDL proteome in T2DM occur early in disease progression, and likely precede diagnosis of T2DM.

Three proteins reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets. Of these, ApoA-I and ApoD were decreased in the combined obesity group, while CF1 was increased in the combined obesity group (**Table *4.7*** & **Figure 4.13 B in Appendix 4D**).

Table 4.7 *Significantly changed proteins on L-HDL-P.*

In T1DM vs Con1/Non-OB	In T2DM vs Con2/OB	In Con2/OB vs Con1/Non-OB	In Con2/OB+T2DM vs Con1/Non-OB
		Increased	
Complement component C8 gamma chain	Transferrin receptor protein 1	Complement C3	Complement component C8 gamma chain
Endoplasmic reticulum chaperone BiP	Beta-Ala-His dipeptidase	Immunoglobulin heavy variable 4/OR15-8(non-fntal)(Frag)	Immunoglobulin heavy variable 4/OR15-8(non-fntal) (Frag)
Lymphatic vessel endothelial hyaluronic acid receptor 1	Coagulation factor V	Vitamin K-dependent protein Z	C4b-binding protein alpha chain
Extracellular superoxide dismutase [Cu-Zn]	Complement component C7	Heparin cofactor 2	Histone H2A
Beta-2-glycoprotein 1	Probable non-functional immunoglobulin heavy variable 3-38	Afamin	Plasma kallikrein
Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Frag)	Keratin, type I cytoskeletal 9	C3/C5 convertase	Afamin
Inter-alpha-trypsin inhibitor heavy chain H3	**Apolipoprotein A-IV**	Complement factor I	Actin, cytoplasmic 1
Adipocyte plasma membrane-associated protein	**Plasma kallikrein**	Vitamin D-binding protein	Heparin cofactor 2
C4b-binding protein alpha chain	Kininogen-1	Fibrinogen alpha chain	Endoplasmic reticulum chaperone BiP
SAA2-SAA4		Alpha-1-acid glycoprotein 2	Alpha-1-acid glycoprotein 2
Apolipoprotein M		Complement C5	Vitamin D-binding protein
		Vitamin K-dependent protein S	Fibrinogen alpha chain
		Serum amyloid P-component	**Complement factor I**
		Carboxypeptidase N catalytic chain	Serum amyloid P-component
		Clusterin	Complement C5
		Apolipoprotein L1	Phosphatidylinositol-glycan-specific phospholipase D
		C4b-binding protein alpha chain	Scavenger receptor cysteine-rich type 1 protein M130
		Histone H2A	Plexin domain-containing protein 2
		Immunoglobulin heavy variable 2-70D	Apolipoprotein L1
		Cartilage oligomeric matrix protein	Immunoglobulin heavy variable 6-1
		Decreased	
Insulin-like growth factor-binding protein complex acid labile subunit	A-kinase anchor protein 9	Apolipoprotein D	Immunoglobulin heavy variable 3-13
Complement C3	Apolipoprotein A-I	Apolipoprotein A-I	Apolipoprotein F
Antithrombin-III	Immunoglobulin kappa variable 2D-29	L-selectin	Serum paraoxonase/lactonase 3
	Alpha-1-antitrypsin	Serum paraoxonase/arylesterase 1	**Apolipoprotein D**
	Immunoglobulin heavy variable 2-70D	Immunoglobulin heavy constant gamma 1	**Apolipoprotein A-I**
	Gelsolin	Immunoglobulin kappa variable 1-33	Apolipoprotein M
	Immunoglobulin heavy variable 3-73	Immunoglobulin lambda constant 3	Serum paraoxonase/arylesterase 1
		Immunoglobulin heavy constant alpha 1	L-selectin
		Immunoglobulin kappa constant	Immunoglobulin heavy constant gamma 1
		Immunoglobulin heavy variable 1-24	Immunoglobulin lambda constant 3
		Haptoglobin-related protein	Immunoglobulin kappa constant
		Immunoglobulin kappa variable 2-24	Haptoglobin-related protein
			Immunoglobulin kappa variable 2-24

All proteins were significant $P<0.05$.
Proteins in bold reached the statistical threshold of the FDR of 5% which adjusts for multiple comparisons in large datasets.

4.3.9 Modulation of the proteomic composition of S-HDL-P

We next identified changes in the pattern of HDL-associated proteins on S-HDL-P between controls and people with T1DM or T2DM (*n*=20 per group).

590 valid proteins were detected on S-HDL-P after MS.

4.3.9.1 Modulation of the proteomic composition of S-HDL-P in T1DM

192 proteins were identified on S-HDL-P from both non-OB and T1DM after filtering for 14 valid proteins (70%) in at least one group. 18 significantly different proteins on S-HDL-P were identified between non-OB and T1DM. Of these, ten HDL-associated proteins were significantly decreased in T1DM on S-HDL-P compared to its non-OB controls, including two Igs**;** alpha-2-HS-glycoprotein (AHSG) which is involved in the acute-phase response; phosphatidylcholine-sterol acyltransferase (LCAT), a HDL remodeling protein; the antioxidant protein PON1; and the apolipoprotein, ApoD. By contrast, eight HDL-associated proteins were significantly increased on S-HDL-P in T1DM compared to non-OB controls including complement C2 (C2), C3/C5 convertase (C3/C5) and complement component C9 (C9), which are involved in the complement system (**Table *4.8*** & **Figure 4.14 A&B in Appendix 4E**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}$=3 in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 4.14 B in Appendix 4E**).

4.3.9.2 Modulation of the proteomic composition of S-HDL-P in T2DM

190 proteins were identified on S-HDL-P from both OB controls and T2DM after filtering for 14 valid proteins (70%) in at least one group. 22 significantly different proteins on S-HDL-P were identified between OB controls and T2DM. Of these, eight HDL-associated proteins were significantly increased on S-HDL-P in T2DM compared to its OB-control, including complement proteins, C3/C5 convertase (C3/C5) and complement component C7 (C7); plasma kallikrein (KLKB1), and coagulation factor IX (F9), which are involved in blood coagulation; alpha-2-antiplasmin (SERPINF2), and alpha-2-HS-glycoprotein (AHSG), which are involved in the acute-phase response; and the apolipoprotein ApoA-IV. By contrast, 14 HDL-associated proteins were significantly decreased on S-HDL-P in T2DM compared to OB-controls of which ten were Igs and others included 72 kDa type IV collagenase which is involved in angiogenesis; coagulation factor XIII B chain (F13B), which is involved in blood coagulation; and the complement protein, complement C4-B (C4B) (**Table *4.8*** & **Figure 4.15 A&B in Appendix 4E**).

Three proteins reached the statistical threshold of the FDR of 5% ($-Log_{10}$=3 in volcano plot), which adjusts for multiple comparisons in large datasets. Of these, Ig kappa variable 2D-28

(IGKV2D-28) was decreased on S-HDL-P in T2DM, while KLKB1 and C3/C5 were increased on S-HDL-P in T2DM (**Table *4.8*** & **Figure 4.15 B in Appendix 4E**).

4.3.9.3 Modulation of the proteomic composition of S-HDL-P in control subjects with obesity relative to age- and sex-matched people without obesity

190 proteins were identified on S-HDL-P from control groups with and without obesity after filtering for 14 valid proteins (70%) in at least one group. 11 significantly different proteins on S-HDL-P were identified between people with and without obesity. Of these, four HDL-associated proteins were significantly decreased on S-HDL-P in obesity compared to the non-obesity group, including Ig lambda-like polypeptide 1 (IGLL1); vitamin K-dependent protein Z (PROZ), which is involved in blood coagulation; and L-selectin (SELL), which is involved in cell adhesion.

By contrast, seven HDL-associated proteins were significantly increased on S-HDL-P in obesity compared to the non-obesity group, four of which are proteins involved in the complement system; as well as heparin cofactor 2 (SERPIND1), which is involved in blood coagulation; and the apolipoproteins, ApoC-III, which is a HDL remodelling protein, and ApoL1 (**Table *4.8*** & **Figure 4.16 A&B in Appendix 4E**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 4.16 B in Appendix 4E**).

4.3.9.4 Modulation of the proteomic composition of S-HDL-P in the combined high BMI group (OB&T2DM) relative to non-obese group

We subsequently combined obesity and T2DM groups together to compare all subjects with high BMI to non-obese counterparts. 190 proteins were identified on S-HDL-P from both non-obese and combined obesity groups after filtering for 70% valid proteins in at least one group. 23 significantly different proteins were identified on S-HDL-P between non-obese and combined obesity groups. Of these, 12 HDL-associated proteins were significantly increased on S-HDL-P in the combined obesity group compared to non-obese group, including the complement proteins, C3/C5 convertase (C3/C5), complement C5 (C5)**,** and complement C3 (C3); heparin cofactor 2 (SERPIND1), plasma kallikrein (KLKB1) and coagulation factor IX (F9), which are involved in blood coagulation; kininogen-1 (KNG1), which is involved in the immune response; vitronectin (VTN), which is involved in cell adhesion, and the apolipoproteins, ApoC-III, and ApoL1.

By contrast, 11 HDL-associated proteins were significantly reduced on S-HDL-P in the combined obesity group compared to non-obese group, six of which are involved in the immune response; and others include L-selectin (SELL) which is involved in cell adhesion; and

vitamin K-dependent protein Z (PROZ), which is involved in blood coagulation and sex hormone-binding protein (SHBG) (**Table *4.8*** & **Figure 4.17 A&B in Appendix 4E**).

Two proteins, SHBG, and C3/C5 reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets. SHBG was decreased in the combined obesity group, while C3/C5 was increased in the combined obesity group (**Table *4.8*** & **Figure 4.17 B in Appendix 4E**).

Table 4.8 *Significantly changed proteins on S-HDL-P.*

In T1DM vs Con1/Non-OB	In T2DM vs Con2/OB	In Con2/OB vs Con1/Non-OB	In Con2/OB+T2DM vs Con1/Non-OB
	Increased		
Complement C2	Complement component C7	Heparin cofactor 2	Ig-like domain-containing protein (Frag)
Alpha-1B-glycoprotein	**Plasma kallikrein**	C3/C5 convertase	Kininogen-1
Ceruloplasmin	Apolipoprotein A-IV	Complement component C6	Heparin cofactor 2
C3/C5 convertase	Alpha-2-antiplasmin	Apolipoprotein C-III	Complement factor I
Complement component C9	**C3/C5 convertase**	Complement C5	**C3/C5 convertase**
Endoplasmic reticulum chaperone BiP	Alpha-2-HS-glycoprotein	Complement C3	Plasma kallikrein
Lumican	Coagulation factor IX	Apolipoprotein L1	Apolipoprotein C-III
Immunoglobulin heavy variable 4-30-2	Selenoprotein P		Complement C5
			Complement C3
			Vitronectin
			Coagulation factor IX
			Apolipoprotein L1
	Decreased		
Serum paraoxonase/arylesterase 1	Immunoglobulin heavy variable 3/OR16-12 (non-fntal) (Frag)	Immunoglobulin lambda-like polypeptide 1	Albumin
Apolipoprotein D	72 kDa type IV collagenase	Vitamin K-dependent protein Z	L-selectin
Immunoglobulin lambda constant 3	Inter-alpha-trypsin inhibitor heavy chain H2	L-selectin	Vitamin K-dependent protein Z
Insulin-like growth factor-binding protein 3	Coagulation factor XIII B chain	Sex hormone-binding globulin	A-kinase anchor protein 9
Insulin-like growth factor-binding protein complex acid labile subunit	Immunoglobulin heavy variable 1/OR15-1 (non-fntal) (Frag)		**Sex hormone-binding globulin**
Afamin	Complement C4-B		Probable non-fntal immunoglobulin heavy variable 3-38
Phosphatidylcholine-sterol acyltransferase	**Immunoglobulin kappa variable 2D-28**		Immunoglobulin heavy constant gamma 1
Immunoglobulin kappa variable 1D-39	Immunoglobulin heavy variable 3-13		Immunoglobulin kappa constant
Transthyretin	Immunoglobulin heavy constant gamma 1		Immunoglobulin lambda-like polypeptide 1
Alpha-2-HS-glycoprotein	Immunoglobulin kappa variable 2-40		Cysteine-rich secretory protein 3
	Immunoglobulin kappa constant		Immunoglobulin lambda variable 3-9
	Immunoglobulin kappa variable 3-20		
	Immunoglobulin heavy variable 4/OR15-8 (non-fntal) (Frag)		
	Immunoglobulin heavy variable 4-28		

All proteins were significant $P<0.05$.
Proteins in bold reached the statistical threshold of the FDR of 5% which adjusts for multiple comparisons in large datasets.

4.3.10 LFQ intensities of proteins of interest on large and small HDL-P across all groups

LFQ intensities were graphed to determine the differences in a selection of proteins of interest on both L-HDL-P and S-HDL-P across all four groups.

ApoA-I, ApoA-II, ApoD and ApoM on L-HDL-P were significantly increased, while ApoL1 on L-HDL-P was significantly decreased in T1DM compared to T2DM. ApoA-I and ApoD on L-HDL-P were significantly increased, while ApoL1 on L-HDL-P was significantly decreased in non-obese controls compared to obese controls. ApoA-I on L-HDL-P was significantly increased, while ApoL1 on L-HDL-P was significantly decreased in non-obese controls compared to people with T2DM. No significant differences were observed between groups for the apolipoproteins on S-HDL-P except for ApoL1 on S-HDL-P, which was significantly increased in people with T2DM compared to people with T1DM and also non-obese controls (**Figure 4.5 A-G**).

No differences were observed for the HDL remodelling proteins across groups on L-HDL-P and S-HDL-P except for ApoC-II on S-HDL-P, which was decreased in people with T1DM compared to people with T2DM. The abundance of HDL-remodelling proteins including LCAT, PLTP and ApoC-II was remarkably lower on S-HDL-P relative to L-HDL-P (**Figure 4.6 A-C**).

The antioxidant protein, ApoA-IV on L-HDL-P, was significantly increased in people with T2DM compared to age-, sex-, and BMI-matched controls, people with T1DM and also non-obese controls. SAA2-4 on L-HDL-P was increased in people with T1DM compared to people with T2DM. Complement factor 1 was increased on L-HDL-P from obesity controls compared to non-obese controls. Complement C3 was increased on L-HDL-P in people with T2DM compared to people with T1DM. The antioxidant protein, PON1 on S-HDL-P, was significantly increased in people with T2DM compared to people with T1DM. C3/C5 convertase and complement C3 on S-HDL-P were increased in people with T2DM compared to people with T1DM and also non-obese controls. Complement C2 was increased in people with T1DM compared to non-obese controls (**Figure 4.7 A-H**). It is notable that the abundance of complement proteins was higher on smaller HDL-P relative to L-HDL-P.

Ig kappa variable 2D-28 on L-HDL-P and S-HDL-P was significantly decreased in T2DM compared to non-obese controls. It was also significantly decreased on S-HDL-P in T2DM compared to age-, sex-, and BMI-matched controls (obese controls). Plasma kallikrein on S-HDL-P was significantly increased in people with T2DM compared to obese controls, people with T1DM and non-obese controls. Heparin cofactor 2 was specifically enriched on S-HDL-P and significantly increased in T2DM compared to non-obese controls while sex hormone binding protein on S-HDL-P was significantly decreased in people with T2DM compared to people with T1DM, and undetectable on L-HDL-P (**Figure 4.8 A-H**).

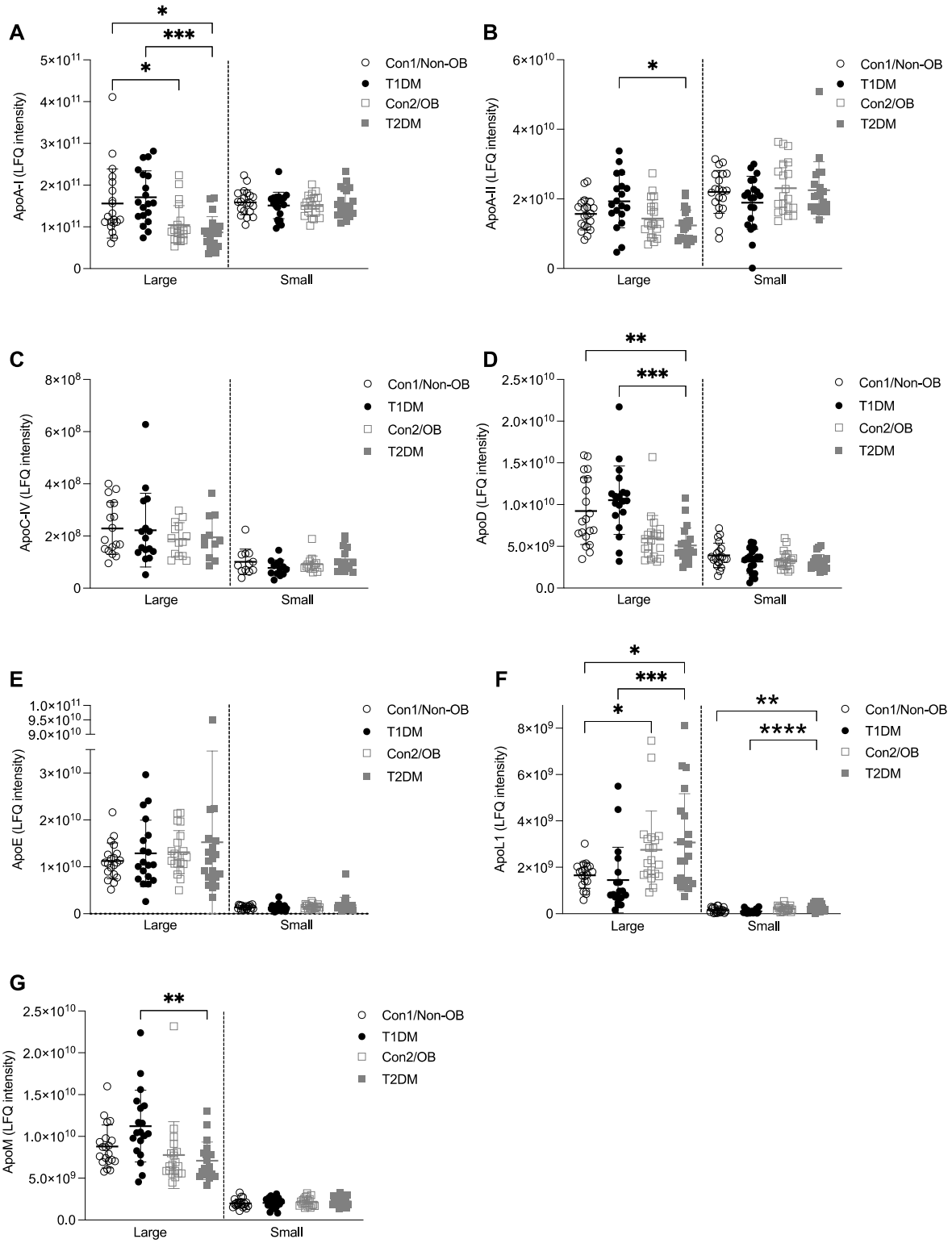


Figure 4.5 *Raw LFQ values of the HDL apolipoproteins on large and small HDL-P across all groups.*

HDL was isolated via FPLC from people with and without T1DM or T2DM (*n*=20 per group). Differences in HDL protein levels on large HDL-P and small HDL-P across all groups were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** ApoA-I, **(B)** ApoA-II, **(C)** ApoC-IV, **(D)** ApoD, and **(E)** ApoE, **(F)** ApoL1, and **(G)** ApoM. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

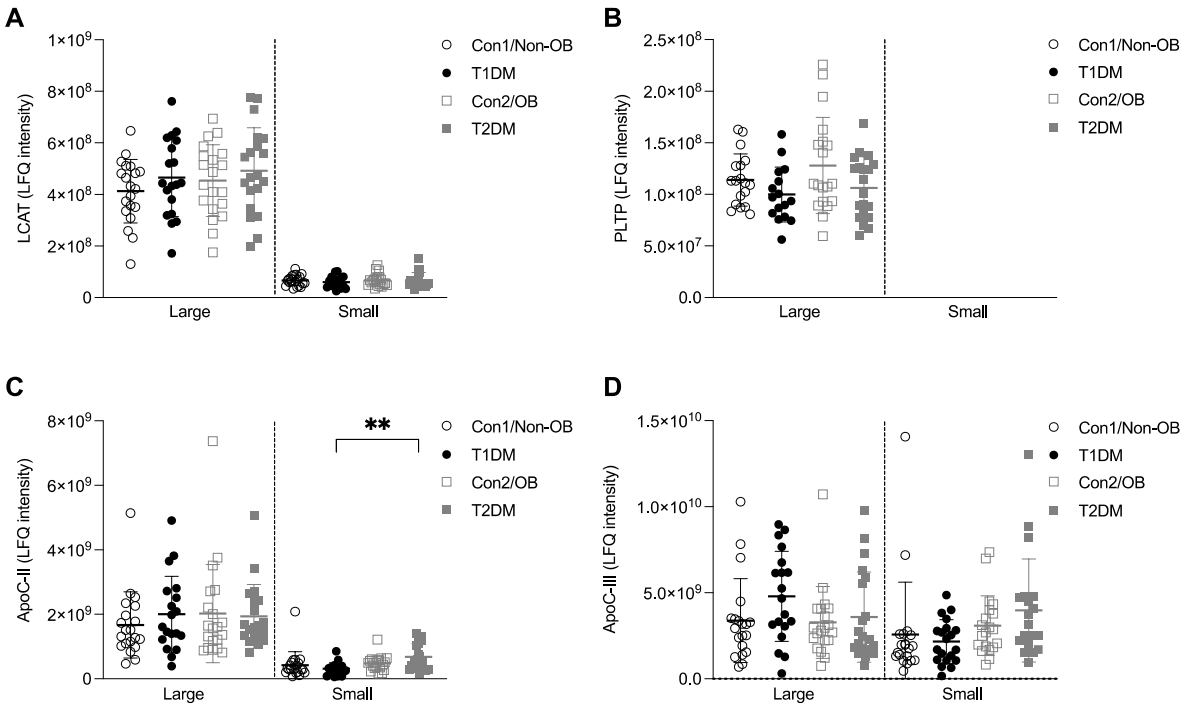


Figure 4.6 *Raw LFQ values of the HDL remodelling proteins on large and small HDL-P across all groups.*

HDL was isolated via FPLC from people with and without T1DM or T2DM (*n*=20 per group). Differences in HDL protein levels on large HDL-P and small HDL-P across all groups were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** LCAT, **(B)** PLTP, **(C)** ApoC-II, and **(D)** ApoC-III. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

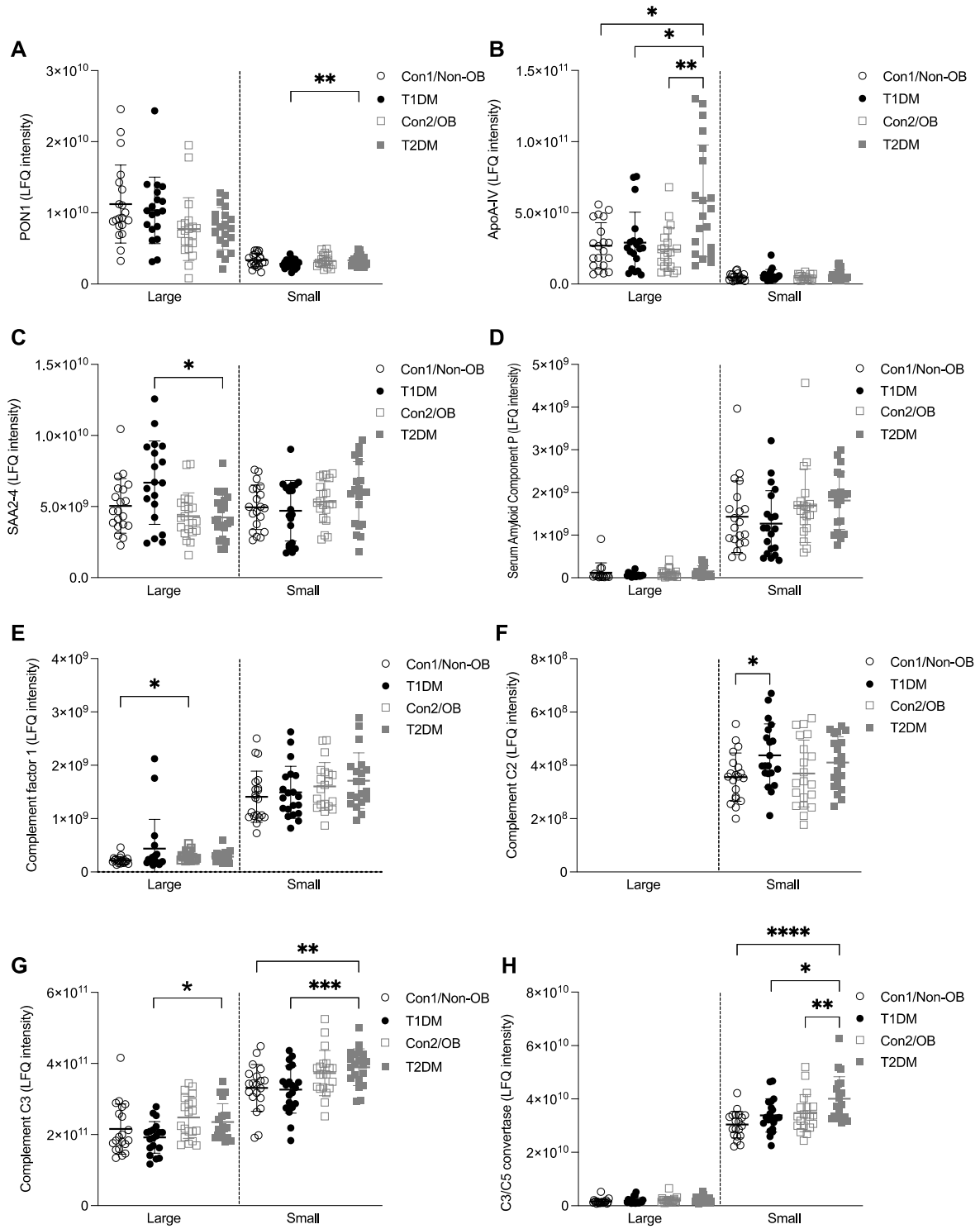


Figure 4.7 *Raw LFQ values of antioxidant, and inflammatory proteins on large and small HDL-P across all groups.*

HDL was isolated via FPLC from people with and without T1DM or T2DM (*n*=20 per group). Differences in HDL protein levels on large HDL-P and small HDL-P across all groups were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** PON1, **(B)** ApoA-IV, **(C)** SAA2-4, **(D)** Serum Amyloid Component P, **(E)** Complement factor 1, **(F)** Complement C2, **(G)** Complement factor C3, and **(H)** C3/C5 convertase. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

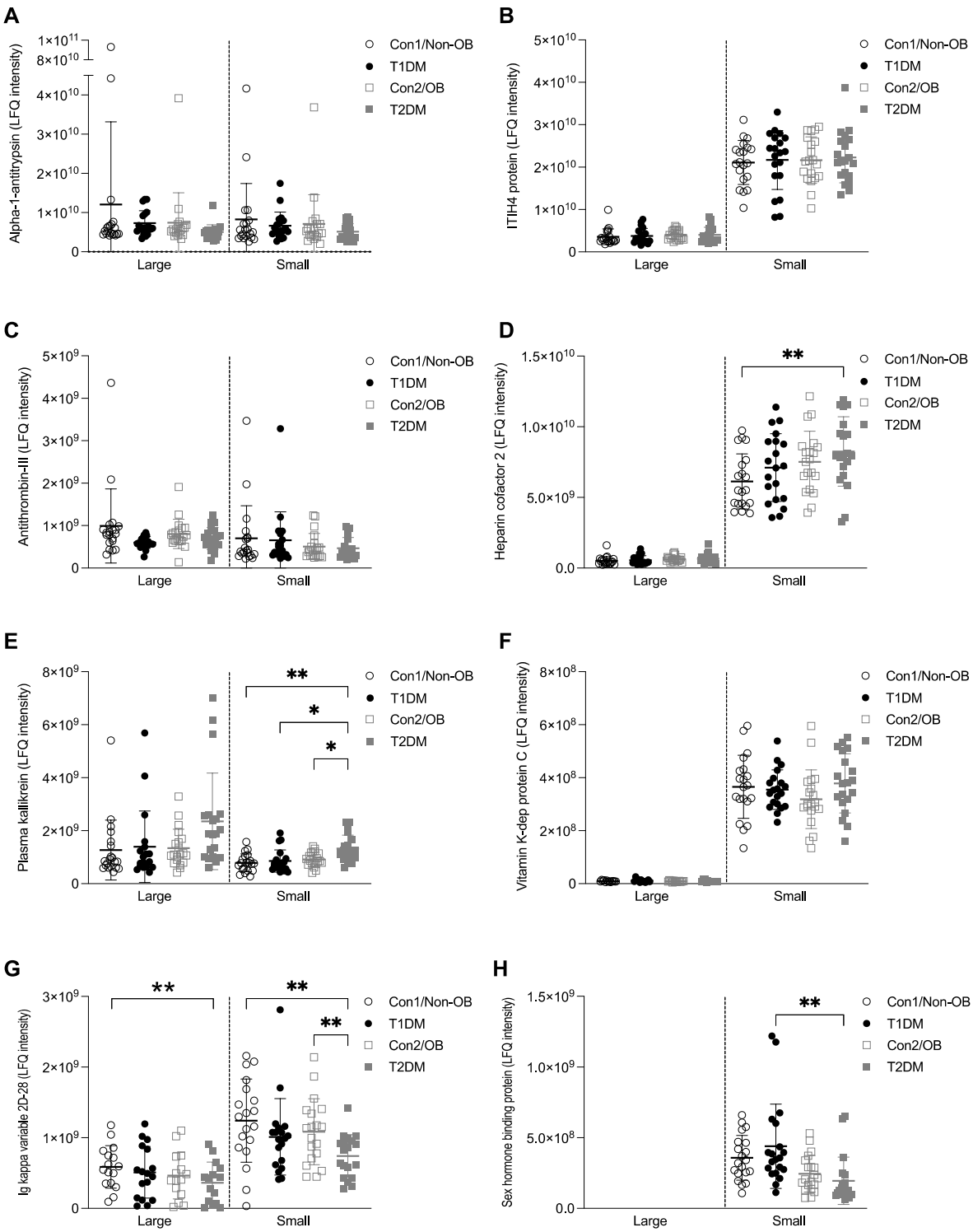


Figure 4.8 *Raw LFQ values of proteins on large and small HDL-P across all groups.*
HDL was isolated via FPLC from people with and without T1DM or T2DM (*n*=20 per group). Differences in HDL protein levels were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** Alpha-1-antitrypsin, **(B)** ITIH4 protein, **(C)** Antithrombin-III, **(D)** Heparin cofactor 2, **(E)** Plasma kallikrein, **(F)** Vitamin K-dep protein C, **(G)** Ig kappa variable 2D-28, and **(F)** Sex hormone binding protein. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

4.3.11 Associations between the proteomic composition of L-HDL-P and total CEC

We next sought to investigate the correlation between HDL-associated proteins and HDL-CEC in people with and without T1DM or T2DM and identify potential protein biomarkers of HDL-CEC that may be unique to people with T1DM or T2DM.

Interestingly, when all groups were combined into one group there were mostly weak correlations between proteins on L-HDL-P and HDL-CEC parameters. Correlations between HDL-associated proteins and HDL-CEC parameters increased to moderate and strong, when separated into the individual groups indicative that proteins governing HDL-CEC in T1DM or T2DM may differ from controls.

Subgroup analysis of the L-HDL-P proteome demonstrated that in non-obese controls, ApoA-II, endoplasmic reticulum chaperone BiP and ITIH4 strongly positively correlated with total CEC, while moderate positive correlations were observed with ApoC-II, ApoM, interleukin accessory protein and kininogen-1. Three proteins, complement 4-B, fibulin-1 and histidine-rich glycoprotein, negatively correlated with total CEC.

In T1DM, none of the apolipoproteins on L-HDL-P correlated with total CEC, which is in contrast to the other groups.

In people with obesity, similar to people without obesity, we observed a strong positive association between ApoA-II and total CEC. Moderate positive correlations were observed between total CEC with ApoA-I and ApoC-III, LCAT and SAA2-4 on L-HDL-P. Three proteins, haemoglobin subunit alpha and haemoglobin subunit beta and phosphatidylinositol-glycan-specific phospholipase D on L-HDL-P, negatively correlated with total CEC in obesity.

In people with T2DM, total CEC strongly positively correlated with alpha-2-HS-glycoprotein, which is involved in the acute phase response, and beta-2-glycoprotein 1 and vitamin-K dependent protein C, which are involved in blood coagulation on L-HDL-P. Moderate negative correlations between Ig-like domain containing protein and von-Willebrand factor on L-HDL-P and total CEC were also observed.

In contrast to the both control groups, there were no significant correlations between, ApoA-II and total CEC on L-HDL-P in T1DM or T2DM.

See **Table *4.9*.**

Table 4.9 *Proteins on large HDL that significantly correlated with total efflux.*

L-HDL associated proteins	All	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
Adipocyte plasma membrane-associated protein	0.303**	0.403	0.154	0.367	0.132
Albumin	0.063	−0.028	−0.076	−0.070	0.497*
Alpha-2-antiplasmin	0.058	0.122	−0.120	−0.275	0.584**
Alpha-2-HS-glycoprotein	0.316**	0.384	−0.052	0.284	0.639**
Antithrombin-III	0.044	0.178	−0.488*	0.241	−0.065
Apolipoprotein A-I	0.225*	0.247	−0.088	0.521*	−0.395
Apolipoprotein A-II	0.454****	0.613**	0.119	0.707***	0.119
Apolipoprotein B-100	0.146	0.489*	0.289	−0.065	0.293
Apolipoprotein C-I	0.309**	0.425	0.083	0.283	0.479*
Apolipoprotein C-II	0.219	0.518*	0.136	−0.020	0.528*
Apolipoprotein C-III	0.336**	0.270	0.268	0.525*	0.094
Apolipoprotein D	0.299**	0.289	0.117	0.385	0.075
Apolipoprotein M	0.314**	0.537*	0.204	0.249	−0.096
Beta-2-glycoprotein 1	0.199	−0.188	0.066	0.062	0.606**
C3/C5 convertase	0.243*	0.307	0.298	0.162	0.422
Cadherin-5	0.194	0.141	0.520*	0.027	0.182
Complement C4-B	−0.230*	−0.487*	0.007	−0.233	−0.027
Complement C5	−0.011	−0.021	0.472*	0.061	−0.150
Complement component C8 alpha chain	0.233*	0.476	0.218	0.118	0.357
Complement factor H	−0.238*	−0.156	−0.105	−0.131	−0.404
Complement factor H-related protein 1	0.257*	0.390	0.281	0.093	0.411
Complement subcomponent C1r	−0.277*	−0.357	−0.314	−0.055	−0.451*
Endoplasmic reticulum chaperone BiP	0.162	0.652**	0.209	−0.215	0.290
Fibulin-1	0.039	−0.509*	−0.027	−0.192	−0.100
Hemoglobin subunit alpha	−0.074	0.091	−0.103	−0.531*	−0.102
Hemoglobin subunit beta	−0.057	0.087	−0.066	−0.483*	−0.065
Hemopexin	0.165	0.284	−0.057	0.007	0.542*
Histidine-rich glycoprotein	0.013	−0.482*	−0.163	0.403	0.189
Ig-like domain-containing protein (Fragment)	−0.138	−0.206	−0.213	0.036	−0.505*
Immunoglobulin heavy constant alpha 2 (Fragment)	0.256*	0.217	0.221	0.300	0.049
Immunoglobulin heavy constant delta	−0.163	−0.165	0.549*	−0.156	−0.281
Immunoglobulin heavy constant gamma 3	−0.247*	−0.325	−0.270	−0.233	−0.429
Immunoglobulin heavy variable 6-1	0.224*	−0.010	−0.033	0.543*	0.351
Immunoglobulin kappa variable 1-6	0.201	0.197	0.510*	0.017	0.121
Immunoglobulin kappa variable 2-24	0.081	0.528*	−0.061	0.111	−0.228
Immunoglobulin lambda variable 3-21	−0.356**	−0.424	−0.404	−0.296	−0.295
Immunoglobulin lambda variable 8-61	0.322**	0.097	0.287	0.446	0.147
Interleukin-1 receptor accessory protein	0.256*	0.556*	0.025	0.331	0.401
ITIH4 protein	0.325**	0.603**	0.139	0.430	0.249
Kininogen-1	0.063	0.488*	−0.361	−0.030	0.139
Phosphatidylcholine-sterol acyltransferase	0.291**	0.362	0.142	0.511*	0.141
Phosphatidylinositol-glycan-specific phospholipase D	−0.143	-0.295	−0.202	−0.478*	0.540*
Plasminogen	0.226*	0.370	0.080	0.168	0.321
Prothrombin	−0.164	0.130	−0.506*	0.156	−0.370
SAA2-SAA4	0.360**	0.254	0.340	0.535*	−0.032
Selenoprotein P	0.225*	0.244	0.221	0.175	0.471*
Vasorin	0.222	-0.352	−0.505*	−0.302	0.167
Vitamin K-dependent protein C	0.134	0.408	−0.182	−0.247	0.636*
Vitamin K-dependent protein S	0.161	0.089	0.477*	0.261	0.125
von Willebrand factor	−0.084	−0.175	0.270	0.182	−0.458*

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.12 Associations between the proteomic composition of L-HDL-P and ABCA1-dependent CEC

Fewer associations between L-HDL-P associated proteins and ABCA1-dependent efflux were evident across all groups.

In non-obese controls, a strong positive correlation was observed between ITIH4 protein and ABCA1-dependent CEC.

Only three proteins on L-HDL-P were significantly associated with T1DM, two of which were Igs that were positively correlated with ABCA1-dependent CEC. ApoA-II on L-HDL-P was strongly associated with ABCA1-dependent CEC, while ApoC-III and ApoD had moderate associations in obese controls. People with T2DM had a strong positive correlation between ApoE and vitamin K-dependent protein C on L-HDL-P, and ABCA1-dependent CEC. Other proteins on L-HDL-P that positively correlated with ABCA1-dependent efflux in T2DM included albumin, alpha-2-HS-glycoprotein, ApoC-I, hemopexin, and phosphatidylinositol glycan-specific phospholipase D. Haptoglobin and protein Z-dependent protease inhibitor on L-HDL-P both significantly negatively correlated with ABCA1-dependent CEC in T2DM.

See **Table 4.10**.

Table 4.10 *Proteins on large HDL that significantly correlated with ABCA1-dependent efflux.*

L-HDL associated proteins	All	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
Albumin	0.197	−0.025	0.314	0.073	0.560*
Alpha-2-HS-glycoprotein	0.277*	0.195	−0.056	0.275	0.551*
Apolipoprotein A-II	0.270*	0.280	−0.049	0.650**	0.082
Apolipoprotein B-100	0.063	0.542*	0.124	−0.321	0.069
Apolipoprotein C-I	0.151	0.079	−0.291	0.170	0.481*
Apolipoprotein C-III	0.274*	0.122	0.177	0.512*	0.128
Apolipoprotein D	0.094	−0.024	−0.270	0.459*	−0.113
Apolipoprotein E	0.173	−0.247	−0.355	0.366	0.613**
Attractin	0.122	−0.145	0.126	−0.235	0.549*
Beta-2-glycoprotein 1	0.186	−0.230	0.129	0.129	0.501*
Ceruloplasmin	0.271*	0.424	0.237	0.132	0.107
Complement C1s subcomponent	−0.262*	−0.291	−0.384	−0.253	−0.018
Complement component C8 beta chain	0.243*	0.136	0.299	0.204	0.401
Endoplasmic reticulum chaperone BiP	0.106	0.558*	0.089	−0.274	0.107
Haptoglobin	−0.006	0.020	0.003	0.090	−0.594**
Hemopexin	0.154	0.183	0.095	0.015	0.576**
Histidine-rich glycoprotein	0.086	−0.514*	0.286	0.428	0.068
Immunoglobulin heavy constant gamma 3	−0.224*	−0.370	0.078	−0.189	−0.431
Immunoglobulin heavy variable 1-69	0.098	0.055	0.558*	−0.017	−0.397
Immunoglobulin heavy variable 3-15	0.013	−0.017	0.586**	−0.150	−0.184
Immunoglobulin lambda variable 3-21	−0.335**	−0.422	−0.326	−0.250	−0.103
Immunoglobulin lambda variable 7-46	0.073	0.033	0.044	0.123	0.503*
Immunoglobulin lambda variable 8-61	0.286*	0.012	0.433	0.442	−0.002
ITIH4 protein	0.340**	0.746***	0.206	0.309	0.119
Kininogen-1	0.130	0.452*	−0.240	0.041	0.220
Phosphatidylinositol-glycan-specific phospholipase D	−0.043	−0.322	−0.009	−0.357	0.543*
Prenylcysteine oxidase 1	−0.069	0.217	−0.470*	0.104	−0.311
Protein Z-dependent protease inhibitor	0.136	−0.001	0.300	0.395	−0.530*
Serotransferrin	0.216	0.072	0.224	0.126	0.462*
Transthyretin	0.265*	0.468*	−0.101	0.182	0.219
Vitamin K-dependent protein C	0.125	0.373	−0.185	−0.092	0.649*

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.13 Associations between the proteomic composition of L-HDL-P and ABCA1-independent CEC

In contrast to ABCA1-dependent CEC, numerous significant correlations between HDL-associated proteins and ABCA1-independent CEC were evident across all groups.

In non-obese controls, strong positive associates between ApoA-I, ApoA-II, ApoC-I, ApoE and ApoM on L-HDL-P and ABCA1-independent CEC were observed. Except for ApoM, these significant correlations were lost in people with T1DM, where we observed remarkably different correlations between the proteome of L-HDL-P and HDL-CEC relative to non-obese controls. Strong positive correlations in T1DM were observed with C4b-binding protein alpha chain, cadherin-5 and SAA2-4, and strong negative correlations between carboxypeptidase N subunit 2 and Ig-like domain-containing protein, on L-HDL-P and ABCA1-independent CEC. Strikingly, negative associations between a suite of Igs (12 in total) on L-HDL-P and ABCA1-independent efflux was evident in T1DM, but not Con1, and may represent important biomarkers of dysfunctional HDL in T1DM.

In obese controls, moderate positive associations between ApoA-I, ApoA-II, and SAA2-4, and a strong association with pregnancy zone protein on L-HDL-P were evident to ABCA1-independent CEC, with a strong negative association to phosphatidylinositol-glycan-specific phospholipase D and haemoglobin subunit alpha.

In T2DM, there were positive correlations between apha-1-acid glycoprotein 2, cadherin-5, ApoC-II and interleukin-1 receptor accessory protein on L-HDL-P with ABCA1-independent CEC. In contrast to T1DM, only one Ig on L-HDL-P was significantly negatively correlated with ABCA1-independent CEC in people with T2DM. The other two proteins on L-HDL-P that negatively correlated with ABCA1-independent CEC were antithrombin-III and prothrombin which are involved in blood coagulation.

See **Table 4.11**.

Table 4.11 *Proteins on large HDL that significantly correlated with ABCA1-independent efflux.*

L-HDL associated proteins	All	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
Adipocyte plasma membrane-associated protein	0.451****	0.349	0.395	0.393	0.495*
Alpha-1-acid glycoprotein 2	0.106	−0.204	−0.102	0.159	0.545*
Alpha-2-macroglobulin	0.155	−0.157	−0.027	0.078	0.487*
Antithrombin-III	−0.136	0.026	−0.482*	−0.034	−0.448*
Apolipoprotein A-I	0.387***	0.604**	0.165	0.515*	−0.085
Apolipoprotein A-II	0.405***	0.630**	0.199	0.474*	0.066
Apolipoprotein A-IV	0.068	0.477*	0.291	−0.106	−0.005
Apolipoprotein C-I	0.319**	0.601**	0.414	0.261	0.120
Apolipoprotein C-II	0.225*	0.363	0.195	0.044	0.464*
Apolipoprotein D	0.378***	0.518*	0.433	0.038	0.345
Apolipoprotein E	0.054	0.776****	0.094	0.080	−0.119
Apolipoprotein F	0.272*	0.443	0.127	−0.115	0.236
Apolipoprotein M	0.499****	0.601**	0.718***	0.203	0.204
Basement membrane-specific heparan sulfate proteoglycan core protein	0.237*	0.377	0.263	0.133	0.455
Beta-Ala-His dipeptidase	0.161	0.199	0.480*	0.117	0.086
C4b-binding protein alpha chain	0.394***	−0.116	0.709***	0.330	0.319
Cadherin-5	0.296**	0.081	0.631**	0.052	0.552*
Carboxypeptidase N subunit 2	−0.197	0.079	−0.629**	-0.015	−0.309
Clusterin	0.074	0.003	−0.149	0.206	0.478*
Coagulation factor X	0.071	−0.067	0.489*	0.229	−0.231
Complement C3	−0.151	−0.569**	0.071	0.398	−0.191
Complement component C7	0.246*	0.090	0.357	0.312	0.008
Complement factor H-related protein 1	0.247*	0.259	0.272	0.047	0.458*
Endoplasmic reticulum chaperone BiP	0.132	0.299	0.162	−0.258	0.475*
GDH/6PGL endoplasmic bifunctional protein	−0.038	0.024	−0.316	−0.011	0.519*
Haptoglobin	−0.066	−0.058	−0.014	−0.482*	0.016
Haptoglobin-related protein	−0.120	−0.228	0.152	−0.473*	0.078
Hemoglobin subunit alpha	−0.091	0.127	−0.097	−0.642**	−0.238
Hemoglobin subunit beta	−0.114	0.111	−0.114	−0.552*	−0.285
Hepatocyte growth factor activator	0.227*	0.152	0.418	0.102	0.409
Histidine-rich glycoprotein	−0.092	-0.076	−0.505*	0.125	0.126
Ig-like domain-containing protein (Fragment)	−0.272	−0.138	−0.625**	−0.129	−0.340
Immunoglobulin heavy constant alpha 1	−0.288*	−0.163	−0.370	−0.298	−0.146
Immunoglobulin heavy constant alpha 2 (Fragment)	0.241*	0.182	0.303	0.202	0.046
Immunoglobulin heavy constant delta	−0.05152	0.062	0.481*	−0.094	−0.182
Immunoglobulin heavy constant gamma 2	−0.030	0.167	−0.482*	0.152	−0.216
Immunoglobulin heavy variable 1-18	−0.239*	−0.028	−0.498*	−0.183	−0.223
Immunoglobulin heavy variable 1-24	−0.076	−0.009	0.063	−0.360	−0.518*
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	0.192	−0.214	0.069	0.449*	0.206
Immunoglobulin heavy variable 3-7	−0.172	0.067	−0.574*	0.007	−0.253
Immunoglobulin heavy variable 3-74	−0.134	−0.066	−0.537*	0.169	−0.080
Immunoglobulin heavy variable 4-28	−0.078	−0.073	−0.489*	0.166	−0.077
Immunoglobulin heavy variable 4-38-2	−0.041	−0.015	−0.548*	0.184	−0.001
Immunoglobulin heavy variable 4-4	−0.060	0.081	−0.469*	0.006	0.046
Immunoglobulin heavy variable 6-1	0.097	−0.090	−0.042	0.504*	0.002
Immunoglobulin kappa variable 1-33	−0.172	−0.191	−0.540*	0.029	−0.194
Immunoglobulin kappa variable 1-6	0.136	0.112	0.517*	−0.081	−0.227
Immunoglobulin kappa variable 2-40	−0.215	−0.198	−0.489*	−0.341	0.186
Immunoglobulin kappa variable 4-1	−0.1713	-0.037	−0.494*	−0.044	−0.168
Immunoglobulin lambda constant 3	−0.257*	−0.095	−0.482*	−0.253	−0.338
Immunoglobulin lambda variable 1-47	−0.048	0.199	−0.502*	0.103	−0.068
Immunoglobulin lambda variable 7-46	0.148	−0.313	0.244	0.493*	0.005
Interleukin-1 receptor accessory protein	0.136	0.501*	−0.369	0.066	0.566*
Keratin, type I cytoskeletal 10	0.277*	0.237	0.395	0.246	0.180
Phosphatidylcholine-sterol acyltransferase	0.332**	0.227	0.406	0.436	0.284
Phosphatidylinositol-glycan-specific phospholipase D	−0.184	−0.038	−0.239	−0.607**	0.269
Plasminogen	0.154	0.139	−0.001	−0.009	0.529*
Pregnancy zone protein	0.243	0.052	0.453	0.665**	0.001
Prenylcysteine oxidase 1	0.444****	0.325	0.596**	0.336	0.517*
Probable non-functional immunoglobulin heavy variable 3-38	−0.094	0.058	−0.457*	0.270	−0.062
Prothrombin	−0.235*	0.256	−0.451	−0.115	−0.446*
SAA2-SAA4	0.515****	0.329	0.605**	0.598**	0.190
Selenoprotein P	0.212	0.240	0.161	0.132	0.523*
Serotransferrin	−0.111	0.221	−0.543*	−0.111	0.125
Serum paraoxonase/lactonase 3	0.329**	0.422	0.342	0.269	0.146
Vasorin	−0.198	−0.181	−0.596**	-0.220	0.279
Vitronectin	−0.259*	-0.141	−0.491*	−0.070	−0.260

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

4.3.14 Associations between the proteomic composition of S-HDL-P and total CEC

Similar to associations between the proteomic composition of L-HDL-P and HDL-CEC parameters, the proteomic composition of S-HDL-P and efflux parameters was weaker when all groups were combined.

In non-obese controls, a strong positive association between Ig heavy variable 3-74 on S-HDL-P and moderate associations between ApoB-100, fibulin-1 and kininogen-1 on S-HDL-P, and total CEC were observed. Negative correlations between ApoL1 and complement C1s subcomponent on S-HDL-P and total CEC were also observed.

The associations between S-HDL-P proteins and total CEC in T1DM remained profoundly different to the non-obese group, consistent with findings on L-HDL-P. In particular, positive associations between complement component 8 alpha, beta and gamma chains, C3/C5 convertase and complement component 9 on S-HDL-P and total CEC were evident in T1DM.

In the obese control group, haemoglobin subunit alpha on S-HDL-P strongly negatively associated with total CEC. In people with T2DM, there were significant correlations between the apolipoproteins on S-HDL-P and total CEC, which were not apparent in their obese control counterparts. Most notably, there were positive correlations between ApoC-I, ApoC-II, ApoC-III, ApoD, ApoE and ApoM on S-HDL-P with total CEC in T2DM. Positive correlations were also observed between adipocyte plasma membrane-associated protein, tetranectin, and the complement proteins, C3, C5 and component C7 on S-HDL-P with total CEC in T2DM.

See **Table 4.12**.

Table 4.12 *Proteins on small HDL that significantly correlated with total efflux.*

S-HDL associated proteins	All	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
Adipocyte plasma membrane-associated protein	0.157	0.089	−0.071	0.162	0.685**
Albumin	−0.047	−0.202	−0.008	0.162	−0.477*
Apolipoprotein B-100	0.223*	0.510*	0.210	−0.160	0.419
Apolipoprotein C-I	0.036	−0.081	−0.021	0.166	0.667**
Apolipoprotein C-II	0.127	0.099	0.032	−0.064	0.582**
Apolipoprotein C-III	0.084	0.115	0.308	0.298	0.584**
Apolipoprotein D	−0.018	−0.203	0.066	−0.227	0.455*
Apolipoprotein E	0.061	−0.030	−0.201	−0.219	0.481*
Apolipoprotein L1	−0.113	−0.461*	−0.027	−0.172	0.199
Apolipoprotein M	0.069	−0.337	0.159	−0.103	0.576**
C3/C5 convertase	0.019	−0.117	0.458*	0.134	0.315
Clusterin	0.139	0.427	−0.114	0.173	0.483*
Coagulation factor X	0.218	0.379	0.140	0.203	0.571**
Coagulation factor XIII B chain	0.050	0.207	−0.027	0.026	0.530*
Complement C1s subcomponent	−0.037	−0.464*	−0.212	0.070	−0.097
Complement C3	−0.108	−0.158	0.056	−0.195	0.516*
Complement C5	−0.029	-0.120	0.047	0.018	0.516*
Complement component C7	−0.076	0.008	−0.612**	0.199	0.533*
Complement component C8 alpha chain	0.129	0.175	0.640**	−0.114	−0.162
Complement component C8 beta chain	0.124	0.132	0.686***	−0.074	−0.071
Complement component C8 gamma chain	0.020	−0.011	0.677**	−0.301	−0.319
Complement component C9	0.120	0.191	0.586**	0.139	−0.110
Complement factor H-related protein 1	0.144	0.467*	0.355	0.059	0.451*
Cysteine-rich secretory protein 3	−0.041	0.231	−0.354	−0.524*	−0.108
EGF-containing fibulin-like extracellular matrix protein 1	−0.067	−0.211	0.460*	0.126	0.381
Fibrinogen alpha chain	−0.182	−0.026	0.467*	0.094	0.064
Fibulin-1	0.243	0.489*	0.163	−0.053	−0.215
Glutathione peroxidase	0.118	0.453*	0.360	0.050	0.308
Hemoglobin subunit alpha	0.112	0.198	−0.068	−0.618*	0.026
Hepatocyte growth factor activator	0.207	0.446*	0.001	−0.155	0.115
Immunoglobulin heavy constant gamma 3	−0.146	−0.266	−0.576**	0.114	−0.254
Immunoglobulin heavy constant mu	0.112	0.402	−0.319	0.147	0.495*
Immunoglobulin heavy variable 1-69	0.160	0.461*	−0.046	0.056	0.179
Immunoglobulin heavy variable 3-7	0.032	0.288	0.022	0.457*	−0.346
Immunoglobulin heavy variable 3-74	0.100	0.639**	−0.016	0.135	−0.334
Immunoglobulin heavy variable 4-38-2	0.346**	0.453*	0.135	0.131	0.044
Immunoglobulin heavy variable 5-51	0.229*	0.276	0.163	0.137	0.113
Immunoglobulin heavy variable 6-1	0.160	0.538*	−0.211	0.483*	0.167
Immunoglobulin kappa variable 1-17	−0.074	0.142	−0.108	0.566**	−0.075
Immunoglobulin kappa variable 1-33	0.225*	0.218	−0.083	−0.066	0.110
Immunoglobulin lambda variable 4-69	−0.152	0.090	0.042	0.130	−0.539*
Immunoglobulin lambda variable 5-45 (Fragment)	−0.228*	−0.061	−0.570*	−0.232	−0.138
Inter-alpha-trypsin inhibitor heavy chain H1	0.230*	0.129	−0.081	0.004	−0.077
Inter-alpha-trypsin inhibitor heavy chain H3	0.248*	0.070	0.022	0.083	−0.328
Kallistatin	0.188	−0.082	−0.155	0.479*	−0.047
Keratin, type II cytoskeletal 1	0.227*	0.554*	0.226	0.059	0.335
Kininogen-1	0.238*	0.495*	−0.102	0.500*	0.217
L-selectin	0.037	0.056	−0.531*	−0.118	0.183
Low affinity immunoglobulin gamma Fc region receptor III-A	−0.061	0.125	−0.195	−0.524*	0.353
Phosphatidylcholine-sterol acyltransferase	−0.037	−0.243	−0.460	−0.361	0.464*
Phosphatidylinositol-glycan-specific phospholipase D	0.037	0.079	−0.211	0.248	0.462*
Plasma kallikrein	0.022	0.252	0.231	0.152	0.567**
Platelet factor 4	0.293*	0.189	−0.041	0.186	0.427
Probable non-functional immunoglobulin heavy variable 3-38	0.292*	0.457*	0.519*	−0.041	0.242
Protein AMBP	0.097	0.226	−0.231	0.087	0.503*
Protein Z-dependent protease inhibitor	0.265*	0.306	0.491*	0.044	0.077
Serum amyloid P-component	0.081	0.371	0.203	−0.283	0.493*
Tetranectin	0.111	0.086	0.010	−0.097	0.627**
Vitamin K-dependent protein C	0.109	0.068	0.259	0.334	0.467*
Vitamin K-dependent protein S	0.082	−0.241	0.098	0.123	0.566**

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.15 Associations between the proteomic composition of S-HDL-P and ABCA1-dependent CEC

Relatively fewer proteins on S-HDL-P correlated with ABCA1-dependent CEC.

In non-obese controls, similar to total CEC, there was a strong positive correlation between Ig heavy variable 3-74 on S-HDL-P and ABCA1-dependent CEC, while a strong positive correlation existed between Ig heavy variable 2-26 on S-HDL-P and ABCA1-dependent CEC was evident in people with T1DM.

Only four proteins on S-HDL-P were significantly associated with ABCA1-dependent in the obese control group. Beta-2-glycoprotein 1 was positively associated, while complement factor H, hemoglobulin subunit alpha and low affinity Ig gamma Fc region receptor III-A were negatively associated with ABCA1-dependent CEC.

In people with T2DM there were strong positive associations between ApoC-I, ApoC-III, ApoE and plasma kallikrein; and moderate associations between ApoC-II and ApoM on S-HDL-P and ABCA1-dependent CEC. Negative associations between haptoglobin, haptoglobin-related protein and hemopexin on S-HDL-P and ABCA1-dependent CEC were also evident in T2DM. See **Table 4.13**.

Table 4.13 *Proteins on small HDL that significantly correlated with ABCA1-dependent efflux.*

S-HDL associated proteins	All	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
Alpha-1-antichymotrypsin	0.240*	0.271	0.335	0.188	−0.266
Apolipoprotein B-100	0.185	0.567**	0.047	−0.307	0.099
Apolipoprotein C-I	−0.040	−0.162	−0.284	0.232	0.633**
Apolipoprotein C-II	0.131	−0.039	−0.012	−0.020	0.495*
Apolipoprotein C-III	0.026	−0.016	−0.107	0.235	0.627**
Apolipoprotein E	0.009	−0.339	−0.167	−0.048	0.640*
Apolipoprotein M	0.047	−0.179	−0.143	−0.034	0.520*
Beta-2-glycoprotein 1	0.007	0.371	0.280	0.510*	−0.216
Beta-Ala-His dipeptidase	−0.098	0.031	0.162	0.086	0.598**
Biotinidase	0.138	0.452*	−0.256	−0.100	0.082
Coagulation factor XII	0.295**	0.287	0.599**	−0.065	−0.403
Coagulation factor XIII B chain	−0.035	0.088	−0.179	0.017	0.575**
Complement C1r subcomponent-like protein	−0.137	−0.159	−0.458*	−0.339	0.003
Complement component C7	−0.178	−0.185	−0.555*	0.216	0.542*
Complement component C8 gamma chain	−0.052	0.145	0.449*	−0.320	−0.268
Complement factor H	0.015	−0.066	0.017	−0.458*	0.343
Complement subcomponent C1r	0.066	0.599**	−0.224	−0.187	−0.305
Fibulin-1	0.376**	0.573*	0.276	−0.091	−0.278
Haptoglobin	0.052	0.073	0.003	−0.137	−0.501*
Haptoglobin-related protein	0.113	0.342	−0.587*	−0.172	−0.619*
Hemoglobin subunit alpha	0.041	−0.126	−0.005	−0.523*	0.218
Hemopexin	0.054	0.005	0.121	−0.075	−0.533*
Hepatocyte growth factor activator	0.242*	0.546*	0.034	−0.133	−0.263
Histidine-rich glycoprotein	−0.035	−0.539*	−0.2945	0.224	0.119
Immunoglobulin heavy constant mu	0.096	0.186	−0.194	0.095	0.551*
Immunoglobulin heavy variable 2-26	0.005	−0.030	0.601*	-0.336	-0.171
Immunoglobulin heavy variable 2-70D	0.078	−0.107	0.590**	-0.077	0.073
Immunoglobulin heavy variable 3-74	0.191	0.621**	0.208	0.145	−0.400
Immunoglobulin heavy variable 4-38-2	0.346**	0.324	0.350	0.013	−0.005
Immunoglobulin heavy variable 5-51	0.223*	0.126	0.511*	−0.121	0.132
Immunoglobulin kappa variable 1-8	0.162	0.000	−0.045	−0.067	−0.483*
Immunoglobulin kappa variable 2D-28	−0.157	−0.495*	0.151	0.073	0.055
Immunoglobulin lambda variable 5-45 (Fragment)	−0.228*	−0.242	−0.447	−0.033	0.037
Immunoglobulin lambda variable 9-49	0.221	−0.583*	0.264	0.291	−0.142
Insulin-like growth factor-binding protein complex acid labile subunit	−0.092	0.175	−0.450*	0.130	−0.194
Kininogen-1	0.114	0.578**	−0.147	0.402	0.004
Low affinity immunoglobulin gamma Fc region receptor III-A	0.024	0.196	−0.310	−0.489*	0.152
Plasma kallikrein	−0.028	0.256	−0.235	0.091	0.600**
Platelet factor 4	0.076	−0.042	−0.446	0.093	0.524*
Tetranectin	0.116	0.036	0.092	0.035	0.519*
Transforming growth factor-beta-induced protein ig-h3	0.228*	0.241	0.132	−0.045	0.289

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4.3.16 Associations between the proteomic composition of S-HDL-P and ABCA1-independent CEC

In contrast to ABCA1-dependent CEC, which exhibited very few correlations to S-HDL-associated proteins, numerous significant correlations between S-HDL-associated proteins and ABCA1-independent CEC were evident.

In non-obese controls, positive correlations between eight Igs on S-HDL-P and ABCA1-independent CEC were evident. A negative correlation between Complement C4-B on S-HDL-P and ABCA1-independent CEC was also evident. No apolipoproteins on S-HDL-P correlated with ABCA1-independent CEC in the control group.

In T1DM, actin cytoplasmic 1, C3/C5 convertase, C4b-binding protein alpha chain, fibrinogen alpha chain, Ig lambda variable 8-61, and protein Z-dependent protease inhibitor on S-HDL-P strongly positively correlated with ABCA1-independent CEC. The majority of proteins on S-HDL-P that negatively correlated with ABCA1-independent CEC were within the Ig family, akin to findings on L-HDL-P.

In obese controls, there was a strong positive association between Ig heavy variable 6-1 on S-HDL-P, and ABCA1-independent CEC, while a strong negative association was observed between LCAT on S-HDL-P and ABCA1-independent CEC. In T2DM, hepatocyte growth factor activator, Ig lambda variable 9-49, keratin type I cytoskeleton 10, and keratin type II cytoskeleton 1 on S-HDL-P strongly positively correlated with ABCA1-independent CEC. Only two proteins on S-HDL-P negatively correlated with ABCA1-independent CEC in T2DM, both of which were Igs.

See **Table 4.14**.

Table 4.14 *Proteins on small HDL that significantly correlated with ABCA1-independent efflux.*

S-HDL associated proteins	All	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
Actin, cytoplasmic 1	0.049	0.311	0.654**	−0.182	−0.206
Adipocyte plasma membrane-associated protein	0.101	0.125	−0.187	0.238	0.594*
Alpha-1-antichymotrypsin	0.042	−0.143	0.082	0.217	0.541*
Alpha-2-antiplasmin	0.174	−0.068	0.124	0.223	0.490*
Angiotensinogen	0.056	−0.313	0.282	0.564**	0.210
Apolipoprotein A-II	−0.041	−0.099	0.450*	−0.036	−0.073
Apolipoprotein B-100	0.128	0.049	0.213	0.041	0.535*
Apolipoprotein C-III	0.106	0.216	0.501*	0.120	−0.017
C3/C5 convertase	0.037	−0.304	0.622**	0.252	0.120
C4b-binding protein alpha chain	0.138	0.026	0.747***	0.194	0.183
Ceruloplasmin	0.216	0.008	0.263	0.519*	−0.241
Coagulation factor X	0.197	0.285	0.402	−0.259	0.574**
Complement C1r subcomponent-like protein	0.042	−0.239	0.049	−0.024	0.492*
Complement C2	0.242*	−0.192	0.435	0.181	−0.137
Complement C3	−0.129	−0.141	0.298	−0.251	0.004
Complement C4-B	0.042	−0.446*	−0.140	−0.323	0.121
Complement component C8 beta chain	0.144	−0.089	0.486*	0.086	0.396
Cysteine-rich secretory protein 3	−0.020	0.223	−0.084	−0.572**	−0.120
Endoplasmic reticulum chaperone BiP	0.268*	0.526*	0.030	0.119	0.351
Extracellular matrix protein 1	0.186	0.259	−0.455*	−0.008	0.121
Fibrinogen alpha chain	−0.129	0.183	0.713***	−0.043	−0.225
Gelsolin	−0.158	−0.334	−0.165	0.176	0.521*
Haptoglobin	−0.065	−0.080	−0.076	−0.553*	−0.090
Hemoglobin subunit alpha	0.141	0.522*	−0.079	−0.529*	−0.342
Hemoglobin subunit beta	0.063	0.553*	−0.059	−0.341	−0.359
Hepatocyte growth factor activator	0.026	−0.029	−0.036	−0.274	0.662**
Hyaluronan-binding protein 2	0.072	−0.184	0.197	0.217	0.580*
Ig-like domain-containing protein (Fragment)	−0.061	0.494*	−0.406	−0.167	−0.351
Immunoglobulin heavy constant gamma 1	−0.158	0.193	−0.674**	−0.192	−0.267
Immunoglobulin heavy constant gamma 3	−0.016	0.042	−0.523*	0.168	−0.226
Immunoglobulin heavy variable 1-18	−0.035	0.401	−0.578**	−0.175	−0.380
Immunoglobulin heavy variable 3-13	−0.013	0.228	0.146	0.026	−0.581*
Immunoglobulin heavy variable 3-72	0.108	0.655**	−0.001	0.421	−0.451*
Immunoglobulin heavy variable 4-4	−0.061	0.392	−0.521*	0.384	-0.032
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	0.074	0.261	−0.496*	−0.336	−0.112
Immunoglobulin heavy variable 6-1	0.075	0.287	−0.045	0.639**	−0.262
Immunoglobulin kappa variable 1-8	0.084	0.511*	0.161	−0.179	−0.165
Immunoglobulin kappa variable 2-30	0.188	0.593**	0.167	0.211	−0.011
Immunoglobulin kappa variable 2-40	−0.086	0.479*	−0.593**	−0.202	−0.203
Immunoglobulin kappa variable 3-20	0.159	0.645**	−0.139	0.136	−0.214
Immunoglobulin kappa variable 6-21	0.056	−0.024	0.560*	−0.074	−0.210
Immunoglobulin lambda constant 3	−0.091	0.374	−0.456*	-0.293	-0.339
Immunoglobulin lambda variable 1-51	0.160	0.640**	−0.220	−0.009	0.008
Immunoglobulin lambda variable 2-14	0.123	0.533*	0.062	−0.073	−0.003
Immunoglobulin lambda variable 3-19	−0.108	−0.044	−0.046	−0.115	0.444*
Immunoglobulin lambda variable 8-61	0.097	0.318	0.632*	0.152	−0.092
Immunoglobulin lambda variable 9-49	−0.104	0.181	−0.350	−0.098	0.723**
Insulin-like growth factor-binding protein 3	0.171	0.580**	−0.158	−0.019	−0.006
Insulin-like growth factor-binding protein complex acid labile subunit	0.283*	0.181	0.191	0.153	−0.023
ITIH4 protein	−0.031	0.199	0.507*	0.126	-0.368
Kallistatin	0.024	−0.249	−0.535*	0.290	0.325
Keratin, type I cytoskeletal 10	0.176	0.596**	0.330	−0.213	0.670**
Keratin, type II cytoskeletal 1	0.224	0.495*	0.470*	0.053	0.697***
Kininogen-1	0.249*	0.009	0.030	0.343	0.285
Phosphatidylcholine-sterol acyltransferase	0.014	−0.076	−0.416	−0.654**	0.398
Plasma kallikrein	0.074	0.059	0.543*	0.096	−0.018
Plasminogen	0.010	−0.149	−0.098	0.525*	0.220
Platelet factor 4	0.406***	0.377	0.499*	0.110	−0.086
Probable non-functional immunoglobulin heavy variable 3-38	0.206	0.619**	0.340	−0.190	−0.075
Protein Z-dependent protease inhibitor	0.236*	0.216	0.642**	−0.067	0.311
Prothrombin	0.234*	0.008	−0.165	−0.179	0.422
SAA2-SAA4	−0.028	−0.336	0.540*	0.158	−0.056
Selenoprotein P	0.182	0.454*	0.134	0.230	0.168
Sex hormone-binding globulin	0.283*	0.462*	0.071	0.387	−0.227
SPARC	0.095	−0.116	0.540*	0.058	−0.107
Vitamin K-dependent protein Z	0.267	0.552*	0.043	0.447	0.220

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

4.4 Discussion

Within this study, we demonstrate for the first time that there are divergent effects of T1DM and T2DM on HDL-P size, efflux capacity and proteomic composition. We demonstrate that the concentration of L-HDL-P increases in T1DM, while the concentration of S-HDL-P decreases relative to non-obese controls; by contrast the concentration of S-HDL-P increases significantly with T2DM relative to T1DM. Total and ABCA1-dependent CEC were significantly higher in T1DM relative to T2DM, with a similar trend evident with ABCA1-independent CEC. HDL-C levels were significantly reduced in T2DM relative to T1DM, and this was a key variable that correlated with ABCA1-independent CEC, but not ABCA1-dependent CEC. The concentration of L-HDL-P positively correlated with total CEC and ABCA1-independent CEC across all groups, with a weaker association to ABCA1-dependent CEC. By contrast, the concentration of S-HDL-P inversely associated with total CEC (except for T2DM, r=0.287) and ABCA1-independent CEC, with little association to ABCA1-dependent CEC across groups with the exception of T2DM where a positive association was evident (r=0.412). Multiple regression revealed the divergent effects of diabetes status on HDL-CEC, with T1DM status associated with an increase in total and specifically ABCA1-independent CEC, while T2DM status was associated with a decrease in total and specifically ABCA1-dependent CEC when controlling for sex, smoking, age, total cholesterol, TG, HDL-C, total HDL-P, HDL size and medications.

Separation of HDL-P into large and small sub-populations for proteomics analysis was critical to identify key proteins associated with the efflux capacity of the particles, with profound differences in the proteomic composition of L-HDL-P and S-HDL-P evident. Despite the positive impact of T1DM on HDL-CEC, the proteome of L-HDL-P was enriched with the pro-inflammatory protein SAA and depleted of the anti-thrombotic protein anti-thrombin-III, relative to BMI-matched control-HDL. T1DM-L-HDL was also enriched with ApoM, which positively correlated with ABCA1-independent efflux (r=0.718), while association of Igs with T1DM-L-HDL inversely correlated with ABCA1-independent efflux and was specific to the T1DM group. The proteome of L-HDL-P in T2DM was depleted of ApoA-I and enriched for ApoA-IV and plasma kallikrein relative to BMI-matched controls. Several proteins modulated in T2DM, were also evident in the obesity group relative to non-obese controls, indicative that changes in the HDL-proteome in T2DM occur early in disease pathogenesis. Interestingly, complement C3 was enriched on L-HDL-P in obesity/T2DM but depleted in T1DM and exhibited a negative correlation to ABCA1-independent efflux in non-obese controls, and thus may represent an important biomarker of HDL dysfunction. Consistent with the lack of association of S-HDL-P with HDL-CEC, we observed much weaker associations between apolipoproteins on S-HDL-

P and HDL-CEC parameters across most groups with the exception of T2DM, where significant correlations between ApoC-I, ApoC-II, ApoC-III, ApoD and ApoE to total and ABCA1-dependent efflux were evident.

Consistent with an important role for L-HDL-P in facilitating ABCA1-independent efflux [18], we demonstrated mostly positive correlations between the concentration of L-HDL-P with total and ABCA1-independent CEC across all groups. HDL-C levels, in turn, were positively associated with total CEC and ABCA1-independent CEC, with no association to ABCA1-dependent efflux evident except for the higher BMI control group. In multiple regression analysis, HDL-C remained significantly positively associated with total CEC when controlling for clinical, biochemical and medication variables. There were weak associations between ABCA1-dependent CEC and L-HDL-P concentration but surprisingly little association to the concentration of S-HDL-P, with the exception of the T2DM group. Despite the shift towards increased concentration of S-HDL-P in T2DM, we observed a significant reduction in ABCA1-dependent efflux relative to both T1DM and lower BMI control groups indicative of dysfunctional particles. This significant reduction in ABCA1-dependent efflux was maintained in T2DM when controlling for clinical, biochemical and medication variables, with T2DM status resulting in a 1.951% decrease in ABCA1-dependent CEC. By contrast, little difference in ABCA1-independent efflux was evident across groups, despite a shift towards increased HDL-P size in T1DM, concurrent with increased concentrations of L-HDL-P, the key acceptors of cholesterol effluxed via ABCG1/SR-B1 pathways. However, T1DM was associated with a 0.818% increase in ABCA1-independent CEC when controlling for clinical, biochemical and medication variables, which was similar to our previous published findings of a 1.42% increase [33]. He *et al.,* similarly observed an impairment in ABCA1-specific efflux in T2DM, while ABCG1-mediated efflux was unimpeded [37]. It is noteworthy in our study that the presence of preβ-1 HDL-P, the dominant mediator of ABCA1-dependent efflux, is not captured in measures of NMR [38], and thus the S-HDL-P captured may not represent the dominant acceptor via ABCA1 accounting for lack of association between S-HDL-P and ABCA1-dependent efflux in our study.

Given the divergent effects of T1DM and T2DM on HDL-P size and HDL-CEC, we opted to characterize the proteome of large and small HDL fractions separately to interrogate the impact of T1DM and T2DM on HDL sub-fractions and in parallel investigate the subsequent relationship to HDL function. We demonstrate remarkable differences in the HDL proteome between L-HDL-P and S-HDL-P. In particular, ApoA-I and ApoA-II, the majority of Igs, complement factor 1, complement C2, complement C3 and C3/C5 convertase were enriched on S-HDL-P while other apolipoproteins including ApoA-IV, ApoC-II, ApoC-III, ApoC-IV, ApoD,

ApoE, ApoL-I and ApoM were enriched on L-HDL-P. The HDL remodelling proteins LCAT and PLTP, the antioxidant PON1, and the inflammatory proteins ITIH1 and ITIH2, were also present in higher abundance on L-HDL-P relative to S-HDL-P. Likewise, Zheng *et al.,* found ApoE, PLTP, LCAT, ApoA-IV, ApoC-III and PON1, enriched on L-HDL-P from human plasma [31]. Zhang *et al.,* have also characterized the proteomic composition of S-HDL-P, M-HDL-P and L-HDL-P, albeit in a mouse model, and similarly found PON1 and ApoE were recruited onto M/L-HDL-P relative to S-HDL-P [39]. Similar to our findings, complement proteins and ApoA-I were reduced, while ApoC-III, ITIH1 and ITIH2 were higher in L-HDL-P relative to S-HDL-P [39]. Our collective data supports a role for both L-HDL-P and S-HDL-P in supporting cholesterol efflux, with an important role for S-HDL-P within the innate immune response, and a more dominant antioxidative role for L-HDL-P.

We found much stronger correlations in this study between the proteome of L-HDL-P and total and ABCA1-independent CEC, relative to the previous chapter where all HDL-containing fractions were pooled into one fraction and weaker associations to efflux parameters were evident. Our findings highlight that L-HDL-P, and its associated proteome, are likely important drivers of HDL-CEC in people with and without T1DM or T2DM. However, our findings also highlight that different proteins are associated with HDL-CEC conditional on metabolic disease status. Within the non-obese control group positive associations between L-HDL-P-associated apolipoproteins including ApoA-I, ApoA-II, ApoA-IV, ApoD, ApoE and ApoM and ABCA1-independent CEC were evident with a significant negative association to complement C3. There were fewer associations between L-HDL-associated proteins and ABCA1-dependent efflux in individuals without obesity, however, a particularly strong positive association between HDL-associated ITIH4 protein and ABCA1-dependent efflux was observed (r=0.746). ITIH4 is a liver produced plasma protein, with poorly characterised functional properties, that is induced during the acute phase response [40]. This strong association between ITIH4 and ABCA1-dependent CEC, however, was only evident in the non-obese control group and is abrogated in T2DM (r=0.119) exemplifying different associations between HDL proteins and HDL-CEC dependent on metabolic disease status.

We identified a number of important changes on the proteome of both L-HDL-P and S-HDL-P in T1DM. L-HDL-P in T1DM were depleted of complement C3, anti-thrombin-III and pro-thrombin and enriched with SAA2-4 and ApoM. In parallel, S-HDL-P were depleted of LCAT, PON1 and ApoD, and enriched with complement C2 and complement component C9. Enrichment with inflammatory-related proteins and depletion of PON1, indicates a shift in the anti-inflammatory profile of HDL-P in T1DM that requires further interrogation. We have previously reported enrichment of HDL with SAA in participants with T1DM [41], and presence

of SAA is thought to displace ApoA-I on HDL-P [42], particularly during the acute phase response [43]. In this study however, we demonstrated a positive association between HDL-associated SAA and ABCA1-independent CEC in T1DM, and no difference in HDL-ApoA-I relative to BMI-matched controls indicative that the level of SAA enrichment in T1DM is not sufficient to displace ApoA-I. SAA is a known acceptor of cellular cholesterol *in vitro* [42], and the present study suggests enrichment of SAA on T1DM-HDL may support HDL-CEC, particularly via ABCA1-independent pathways. Gourgari *et al.,* demonstrated, in youth with T1DM, a significant increase in HDL-associated ITIH4 in people with poorly controlled T1DM, while a reduction in HDL-associated complement C3 was identified only in people with optimal glucose control [44]. We also observed a trend towards increased ITIH4 and a significant reduction in complement C3 on L-HDL-P in people with T1DM relative to BMI-matched controls. Interestingly, Gourgari *et al.,* found no association between HDL-CEC and HDL proteomic composition in their study. This was likely attributable to a combination of only measuring total CEC (no measurement of ABCA1-dependent versus independent CEC) and collating all HDL fractions after FPLC into one pool for proteomics analysis. These findings further support our approach to separate HDL into larger and smaller particles for proteomics analysis and capturing measures of total, ABCA1-dependent and ABCA1-independent CEC.

An interesting observation in our study was the shift in the profile of proteins on L-HDL-P that correlated with HDL-CEC in T1DM relative to non-obese controls. The correlations between ABCA1-independent CEC and ApoA-I, ApoA-II, Apo-IV, ApoC-I, ApoD and ApoE on L-HDL-P evident in non-obese controls were lost, and ApoM (r=0.718) became the dominant apolipoprotein that positively associated with ABCA1-independent CEC in T1DM (with a negative correlation to ABCA1-dependent CEC). ApoM acts as a carrier for sphingosine-1-phosphate (S1P), which enhances the function of the endothelial barrier through stimulation of endothelial nitric oxide (NO) synthase expression [45]. This drives the production of endothelial NO and decreases monocyte activation and adhesion, which have protective abilities against atherosclerosis. Yao *et al.,* have demonstrated that ApoM-enriched HDL mediates greater cholesterol efflux from murine macrophages than ApoM-free HDL, indicative of a functional role for ApoM in driving cellular CEC [46]. These findings indicate that ApoM may have an important role in T1DM for supporting HDL-CEC.

In contrast to ApoM, there was a striking negative correlation between presence of Igs on L-HDL-P in T1DM and ABCA1-independent CEC, that was not evident in other groups. The presence of Igs on HDL is a contentious issue with some believing them to be contaminants of HDL-containing FPLC fractions, as limited numbers are detectable on ultracentrifuged-derived HDL. However, we have included an extra purification step within our HDL-isolation preparation which involves precipitating phospholipid containing particles from FPLC fractions

(i.e. HDL within the fractions) using LRA [47]. Even in the presence of this purification step, Igs were still present and their association with HDL has potentially important detrimental consequences for HDL function in T1DM. We also observed similar negative associations between HDL-Igs on S-HDL-P and ABCA1-independent efflux in T1DM. SAA2-4, ITIH4, fibrinogen alpha chain, C3/C5 convertase and C4b-binding protein alpha chain on S-HDL-P all significantly correlated with ABCA1-independent CEC in T1DM with minimal correlations to ABCA1-dependent efflux evident. There was little correlation between the proteome of S-HDL-P and ABCA1-dependent efflux in T1DM consistent with lack of association of S-HDL-P and ABCA1-dependent CEC within this study. It is however highly likely that the S-HDL fractions chosen were not representative of the lipid-poor preβ-1 HDL-P, which are the dominant acceptor via ABCA1 [48], and are more likely to represent a more medium sized particle given the significant associations to ABCA1-independent efflux.

Similar to T1DM, there were remarkable changes within the HDL proteome of individuals with T2DM relative to both BMI-matched controls (obese controls) and non-obese controls. L-HDL-P exhibited enrichment of ApoA-IV and plasma kallikrein (KLKB1) in T2DM relative to BMI-matched controls. KLKB1 and C3/C5 convertase were significantly enriched, while Ig kappa variable 2D-18 was depleted on S-HDL-P in T2DM relative to BMI-matched controls. KLKB1 on S-HDL-P positively associated with total CEC (r=0.567) and ABCA1-dependent CEC (r=0.600) within the T2DM group only with no association to ABCA1-independent CEC evident (r=−0.018). ApoA-IV on L-HDL-P, by contrast is positively associated ABCA1-independent efflux (r=0.477) in the non-obese control group but no such correlation was evident in T2DM. KLKB1 functions in the kallikrein-kinin systems (KKS). In diabetes, KKS potentially contributes to increased endothelial permeability, glomerular hyperfiltration and glomerular injury but may also play a role in organ protection [49]. Campbell *et al.,* reported increased tissue kallikrein levels in T2DM, consistent with our findings of increased HDL-associated kallikrein [50]. Lindstedt *et al.,* demonstrated that treatment of HDL_3 (S-HDL) with kallikrein for 15 minutes, resulted in a 60% decrease in its' ability to support cholesterol efflux [51]. The authors demonstrated that the protease activity of kallikrein within the HDL-P resulted in cleavage products in HDL_3 particles, but apolipoproteins remained intact. It is therefore plausible that enrichment of HDL with kallikrein in T2DM may be contributing to HDL dysfunction – however the findings from our study suggest that at physiological levels, kallikrein was not associated with impaired HDL-CEC in T2DM, and by contrast was positively associated with CEC.
Cardner *et al.,* previously reported depletion of ApoA-IV, PON1, ApoD, ApoE and ApoM on HDL-P in T2DM and enrichment with serum amyloid proteins, ApoC-II and ApoC-III relative to healthy controls. However, it is notable that their study looked at total HDL-P (large and small combined) isolated via ultracentrifugation and therefore, our results are not directly comparable

[52]. Furthermore, the control group in their study was a non-obese control group (Mean BMI 24 kg/m^2). Our data suggests that these aforementioned findings were a function of obesity within the T2DM group as when we combined our high-BMI groups (obese control and T2DM), we similarly observed decreased ApoD, ApoA-I, PON1/3 and ApoM on L-HDL-P, and enrichment of ApoC-III on S-HDL-P. Sleddering *et al.,* found ApoA-IV was significantly increased in T2DM compared to obese controls, consistent with our findings, however these findings were in plasma and not on HDL directly [53]. He *et al.,* showed no difference in HDL-associated ApoA-IV between people with T2DM and controls but a significant decrease in alpha-1-antitrypsin in S-HDL-P in T2DM compared to controls [37]. Within the current study we observed a significant reduction in alpha-1-antitrypsin on L-HDL in T2DM relative to BMI-matched controls, with a similar trend on S-HDL-P, but this did not reach the significance threshold after adjusting for FDR. Alpha-1-antitrypsin is predominantly produced in the liver and its key functions are protection of pulmonary tissue and regulation of pulmonary immune processes [54]. Similar to our study, He *et al.,* measured ABCA1-CEC and ABCG1-CEC, but specifically used the isolated HDL-P as their acceptor, and correlated changes in efflux to changes in the HDL proteome [37]. They found a positive correlation between alpha-1-antitrypsin on S-HDL-P and ABCA1-CEC, and a negative association between ApoC-II on S-HDL-P and ABCA1-CEC, with a significant reduction in ABCA1-CEC evident in T2DM relative to controls. By contrast, we observed a significant positive correlation between L-HDL-ApoC-II in T2DM to total CEC (r=0.582) and ABCA1-dependent CEC (r=0.633), but not to ABCA1-independent CEC (r=0.238) that was specific for the T2DM group. No association between HDL-CEC and alpha-1-antitrypsin was evident in our study. It is notable that the study by He *et al.,* did not report BMI values for either groups and thus cross comparison is difficult to determine. They also used a different HDL isolation method (ion mobility) and their S-HDL-P were likely smaller than our fractions. He *et al.,* also used a targeted proteomics approach which monitored the 30 most abundant HDL proteins, hence KLKB1 and Igs were not included. Their study also comprised an older population than ours (mean age 61 for controls and 63 in T2DM) that may have further accounted for discrepancies in our findings.

Our study has further highlighted that changes within the HDL proteome occur early in metabolic disease, prior to the onset of T2DM, with the exception of HDL-associated ApoA-IV and KLKB1. In particular, ApoA-I and ApoD were depleted on L-HDL-P and complement factor 1 was enriched on L-HDL-P, in the combined high-BMI/T2DM group relative to non-obese controls. Similarly, C3/C5 convertase was enriched on S-HDL-P, while sex hormone binding globulin (SHBG) was decreased on S-HDL-P within the combined high BMI/T2DM group. C3/C5 convertase (complement factor B) is involved in the alternative complement pathway, which is a component of the innate immune system [55]. The role of this protein on HDL,

however is unknown but it may serve as a biomarker of dysfunctional HDL in the metabolic disease setting. SHBG levels have been shown to negatively associate with adiposity and chronic inflammation in prepubertal children [56]. A retrospective study of 141 men found that decreased SHBG levels were linked to a higher prevalence of metabolic syndrome [57]. Additionally, Ding *et al.,* reported that SHBG levels were negatively correlated with a risk of T2DM in men and women [58]. While all these studies measured the blood plasma/serum levels of SHBG, our study complements these published findings and supports a role for SHBG as a biomarker of metabolic dysfunction.

Similar to findings with T1DM, the proteins associated with HDL-CEC were remarkably modulated in T2DM relative to both their BMI-matched control group and non-obese control group. Strong positive correlations between alpha-2-HS-glycoprotein, beta-2-glycoprotein 1, and vitamin-K-dependent protein C on L-HDL-P and total CEC were evident, with moderate positive correlations to ApoC-II, ApoC-III, and significant negative correlations to complement subcomponent C1r and von Willebrand factor. ApoE and vitamin K-dependent protein C on L-HDL-P were strong positive determinants of ABCA1-dependent efflux in the T2DM group only, with a significant negative association to haptoglobin. Alpha-1-glycoprotein 2, ApoC-II, clusterin and interleukin-1 receptor accessory protein on L-HDL-P all positively correlated with ABCA1-independent efflux, with negative correlations to pro-thrombin, anti-thrombin III and Ig variable 1-24 evident. The strong correlations between ApoA-I (r=0.604) and ApoA-II (r=0.630) on L-HDL-P and ABCA1-independent efflux that was evident in non-obese controls, were completed abrogated in T2DM (r=−0.085 and 0.066 respectively) similar to findings in T1DM (r=0.165 and r=0.199). Interestingly, this loss of correlation was specific for the diabetes groups and not evident in the obesity group where significant correlations to ApoA-I (r=0.515) and ApoA-II (r=0.474) were retained. Post-translational modifications of ApoA-II are evident in T2DM, in particular oxidation [59]. Oxidation of ApoA-I, in turn, is associated with a loss of cholesterol efflux capacity via the ABCA1 pathway [60]. Additionally, glycation of ApoA-I in T2DM is negatively correlated with ABCA1-dependent CEC [61]. It is therefore plausible in our study that modifications in the proteoform of ApoA-I and ApoA-II in people with T1DM or T2DM may have impacted on functionality – this will be an important avenue to address in the future.

In general, there were weaker associations between the apolipoproteins on S-HDL-P and HDL-CEC in the non-obese control group. However, consistent with a shift towards increased concentration of S-HDL-P in T2DM, much greater correlations between the apolipoproteins on S-HDL-P and CEC were evident in T2DM; in particularly strong and moderate associations between ApoC-I, ApoC-II, ApoC-III, ApoD and ApoE on S-HDL-P and total CEC were evident. These proteins were particularly associated with ABCA1-dependent CEC, but not ABCA1-

independent CEC, in T2DM. These findings highlight an important shift, not only in HDL-P size and function in T2DM, but also a shift in the prominent proteins governing HDL-CEC. It will be important in future work to untangle proteins that serve as biomarkers of HDL-CEC from proteins that are causally implicated in HDL-CEC, which is a major challenge for the HDL field given the complexity of these particles.

The study includes the following strengths: (1) inclusion of both, T1DM and T2DM, diseases as well as respective age-, sex and BMI-matched controls in 81 participants per group for CEC and HDL-size analysis; (2) analysis of total, ABCA1-dependent and ABCA1-independent CEC and corresponding HDL-P size and concentration via NMR analysis; (4) profiling of both the large and small HDL proteome in 80 individuals and subsequent correlation analysis to the three different HDL-CEC parameters; and (5) detailed characteristics of participants including smoking status and medications. An important limitation of this study, like the last chapter, is the inability to make causal inference. This study was cross-sectional; therefore, it was not designed to determine the longitudinal effect of T1DM or T2DM on HDL-P function and/or composition and subsequent incidence or progression of CVD. Future, follow-up studies would be needed to evaluate this. Another limitation of this study was that we did not characterise the post-translational modifications within the HDL proteome which may be an important determinant driving HDL dysfunction in T1DM and T2DM.

In conclusion, our findings demonstrate profound differences in HDL biology between T1DM and T2DM with opposing effects on HDL-CEC and particle size. We also demonstrate that the relationship between HDL-associated proteins with HDL efflux capacity is markedly modulated in both T1DM and T2DM relative to non-obese controls. A loss of association between ApoA-I and ApoA-II on L-HDL-P with ABCA1-independent efflux capacity was evident in both T1DM and T2DM. Finally, we demonstrate very little associations between S-HDL-P size, or the apolipoproteins on S-HDL-P, with HDL-CEC, with the exception of the T2DM group where strong correlations between S-HDL-P size and apolipoproteins on S-HDL-P with total CEC and ABCA1-dependent CEC were evident. These findings indicate there are potentially compensatory mechanisms at play in T2DM to overcome loss of function of S-HDL-P. Our findings highlight the importance of capturing proteomic composition of HDL subfractions, as well as measures of total, ABCA1-dependent and ABCA1-independent CEC to decipher HDL structure-function relationships. Further research will be required to tease apart HDL-proteins causally associated with HDL dysfunction from those that are merely correlative.

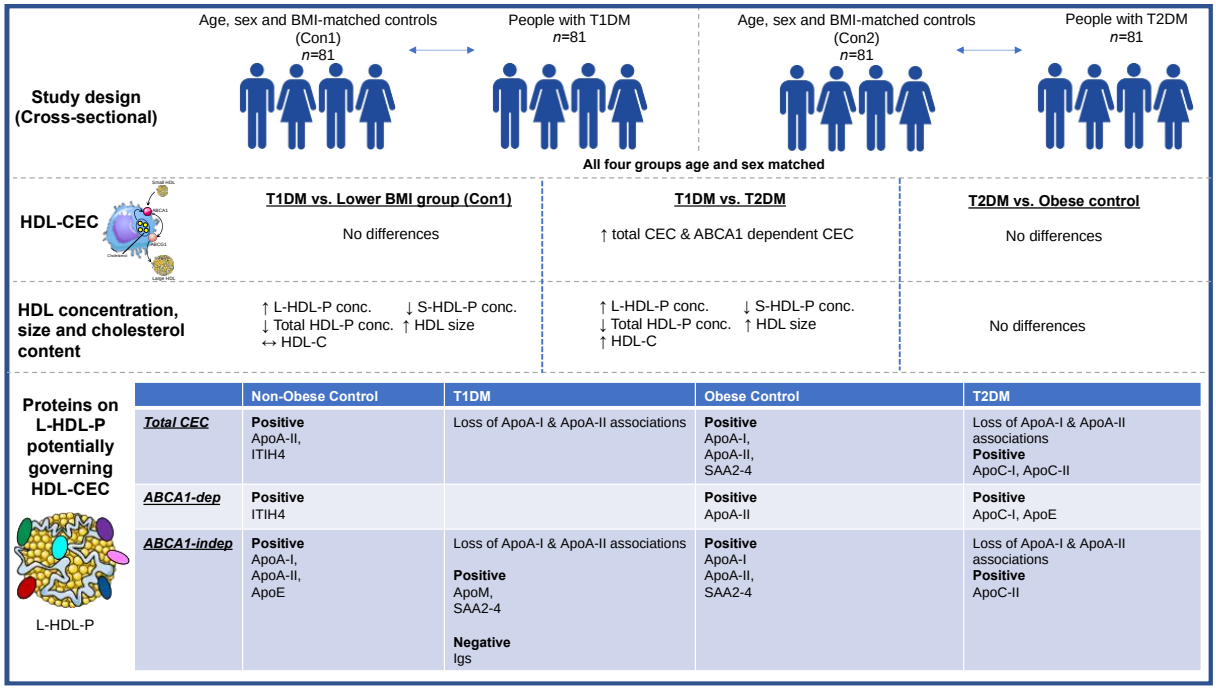


Figure 4.9 *Overall findings of chapter/Graphical abstract.*

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Appendix 4A – Medication tables

Table 4.15 *Medication use in T1DM and T2DM and their respective controls.*

	Con1 (*n*=81)	T1DM (*n*=81)	Con2 (*n*=81)	T2DM (*n*=81)
	%	%	%	%
Medication use overall	1.2	37	1.2	75.3
Medication				
ACE-I/ARB (anti-hypertensive)	0	33.3	0	14.8
Gliclazide (insulin-sensitising)	0	1.2	0	21
DPP4 (insulin-sensitising)	0	1.2	0	12.3
Metformin (insulin-sensitising)	0	1.2	0	68
GLP-1	0	0	0	17.3
Statin	1.2▪	32.1	1.2▪	21

▪Persons on statins oldest in respective groups (Age 59 & 61)

Table 4.16 *Medication use in subcohort of T1DM and T2DM and their respective controls.*

	Con1 (Non-OB) (*n*=20)	T1DM (*n*=20)	Con2 (OB) (*n*=20)	T2DM (*n*=20)
	%	%	%	%
Medication use overall	0	25	5	80
Medication				
ACE-I/ARB (anti-hypertensive)	0	20	0	20
Gliclazide (insulin-sensitising)	0	0	0	30
DPP4 (insulin-sensitising)	0	0	0	15
Metformin (insulin-sensitising)	0	0	0	70
GLP-1	0	0	0	25
Statin	0	25	5▪	20

▪Persons on statins oldest in respective groups (Age 59)

Appendix 4B – All proteins detected on L-HDL-P

216 proteins were detected on L-HDL-P and are shown below.

Table 4.17 *Proteins detected on large HDL-P across groups.*

Protein names	Majority protein IDs	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
	Uniprot	Mean LFQ Intensity			
A-kinase anchor protein 9	A0A2R8Y590	1.22E+08	1.16E+08	1.29E+08	9.29E+07
Actin, cytoplasmic 1	P60709	6.40E+07	8.69E+07	8.64E+07	7.72E+07
Adipocyte plasma membrane-associated protein	Q9HDC9	2.61E+08	5.33E+08	3.75E+08	4.03E+08
Afamin	P43652	4.33E+08	3.65E+08	5.25E+08	5.02E+08
Albumin	P02768	6.77E+10	7.03E+10	7.02E+10	6.49E+10
Alpha-1-acid glycoprotein 2	P19652	1.61E+08	2.05E+08	2.53E+08	2.41E+08
Alpha-1-antichymotrypsin	P01011	1.38E+09	1.44E+09	1.47E+09	1.34E+09
Alpha-1-antitrypsin	P01009	1.21E+10	7.29E+09	7.41E+09	5.06E+09
Alpha-1B-glycoprotein	P04217	5.72E+08	5.83E+08	5.97E+08	6.32E+08
Alpha-2-antiplasmin	P08697	6.60E+08	6.44E+08	6.00E+08	6.40E+08
Alpha-2-HS-glycoprotein	P02765	7.95E+08	8.29E+08	8.59E+08	8.43E+08
Alpha-2-macroglobulin	P01023	7.63E+09	8.35E+09	6.95E+09	7.50E+09
Angiotensinogen	A0A7P0T9S6	2.69E+08	2.24E+08	2.33E+08	2.23E+08
Antithrombin-III	P01008	9.92E+08	6.01E+08	8.03E+08	6.97E+08
Apolipoprotein A-I	P02647	1.56E+11	1.71E+11	1.04E+11	8.65E+10
Apolipoprotein A-II	P02652	1.57E+10	1.93E+10	1.44E+10	1.24E+10
Apolipoprotein A-IV	P06727	2.68E+10	2.91E+10	2.42E+10	5.83E+10
Apolipoprotein B-100	P04114	8.53E+08	8.16E+08	9.82E+08	9.62E+08
Apolipoprotein C-I	K7ERI9	1.43E+09	1.77E+09	1.76E+09	1.43E+09
Apolipoprotein C-II	K7ER74	1.67E+09	2.01E+09	2.02E+09	1.94E+09
Apolipoprotein C-III	P02656	3.39E+09	4.79E+09	3.28E+09	3.59E+09
Apolipoprotein C-IV	P55056	2.29E+08	2.22E+08	1.87E+08	1.95E+08
Apolipoprotein D	P05090	9.23E+09	1.05E+10	5.94E+09	5.10E+09
Apolipoprotein E	P02649	1.13E+10	1.29E+10	1.31E+10	1.52E+10
Apolipoprotein F	Q13790	4.08E+08	6.00E+08	2.83E+08	2.72E+08
Apolipoprotein L1	O14791	1.65E+09	1.45E+09	2.75E+09	3.07E+09
Apolipoprotein M	O95445	8.80E+09	1.12E+10	7.77E+09	7.08E+09
Attractin	O75882	1.43E+09	1.74E+09	1.64E+09	1.89E+09
Basement membrane-specific heparan sulfate proteoglycan core protein	P98160	2.42E+07	2.72E+07	3.07E+07	2.89E+07
Beta-2-glycoprotein 1	P02749	3.23E+08	4.18E+08	3.72E+08	3.12E+08
Beta-Ala-His dipeptidase	Q96KN2	6.71E+08	6.37E+08	6.23E+08	7.77E+08
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	Q9H3R1	8.20E+08	8.85E+08	7.20E+08	5.93E+08
C3/C5 convertase	B4E1Z4	1.59E+09	1.86E+09	2.05E+09	2.00E+09
C4b-binding protein alpha chain	P04003	4.74E+08	6.32E+08	5.63E+08	5.68E+08
Cadherin-13	P55290	1.19E+08	1.17E+08	1.25E+08	1.05E+08
Cadherin-5	P33151	6.65E+08	6.58E+08	6.03E+08	5.87E+08
Carboxypeptidase N catalytic chain	P15169	5.73E+08	5.63E+08	8.27E+08	7.23E+08
Carboxypeptidase N subunit 2	P22792	1.06E+09	8.98E+08	1.18E+09	1.01E+09

Cartilage acidic protein 1	Q9NQ79	2.35E+08	1.95E+08	2.02E+08	1.98E+08
Cartilage oligomeric matrix protein	G3XAP6	5.24E+08	5.50E+08	6.64E+08	5.70E+08
Cathelicidin antimicrobial peptide	P49913	4.86E+07	5.00E+07	4.74E+07	5.42E+07
CD5 antigen-like	O43866	1.33E+08	1.23E+08	9.70E+07	1.14E+08
Cell surface glycoprotein MUC18	P43121	8.87E+07	1.01E+08	9.00E+07	9.33E+07
Ceruloplasmin	P00450	8.39E+09	9.43E+09	7.99E+09	7.40E+09
Clusterin	P10909	1.03E+10	9.94E+09	1.22E+10	1.22E+10
Coagulation factor V	P12259	6.50E+07	8.45E+07	8.00E+07	8.25E+07
Coagulation factor X	P00742	1.02E+08	1.03E+08	1.08E+08	1.37E+08
Coagulation factor XII	P00748	1.30E+08	8.80E+07	6.00E+07	6.94E+07
Coagulation factor XIII B chain	P05160	2.83E+09	2.89E+09	2.90E+09	2.83E+09
Complement C1q subcomponent subunit B (Fragment)	A0A0A0MSV6	1.25E+08	9.66E+07	1.31E+08	1.44E+08
Complement C1q subcomponent subunit C	P02747	1.51E+08	1.49E+08	1.72E+08	1.73E+08
Complement C1r subcomponent-like protein	Q9NZP8	7.71E+07	9.49E+07	1.16E+08	9.49E+07
Complement C1s subcomponent	P09871	1.99E+10	2.05E+10	1.90E+10	1.95E+10
Complement C3	P01024	2.16E+11	1.92E+11	2.49E+11	2.35E+11
Complement C4-A	P0C0L4	1.69E+09	1.89E+09	1.75E+09	2.05E+09
Complement C4-B	P0C0L5	1.87E+11	1.81E+11	2.04E+11	2.26E+11
Complement C5	P01031	2.10E+10	2.20E+10	2.61E+10	2.76E+10
Complement component C6	P13671	1.01E+10	9.83E+09	1.10E+10	1.04E+10
Complement component C7	P10643	3.65E+09	4.09E+09	3.49E+09	3.98E+09
Complement component C8 alpha chain	P07357	5.55E+07	5.08E+07	5.85E+07	5.66E+07
Complement component C8 beta chain	P07358	6.97E+06	1.86E+07	1.18E+07	1.03E+07
Complement component C8 gamma chain	P07360	1.12E+07	1.84E+07	1.73E+07	1.74E+07
Complement component C9	P02748	4.60E+07	5.47E+07	6.64E+07	6.95E+07
Complement factor H	P08603	6.47E+09	6.13E+09	7.45E+09	7.42E+09
Complement factor H-related protein 1	Q03591	1.57E+08	1.88E+08	1.95E+08	1.96E+08
Complement factor H-related protein 2	P36980	4.15E+07	5.81E+07	4.12E+07	4.21E+07
Complement factor I	G3XAM2	2.18E+08	4.38E+08	3.01E+08	2.86E+08
Complement subcomponent C1r	A0A3B3ISR2	1.69E+09	1.56E+09	1.76E+09	1.69E+09
Endoplasmic reticulum chaperone BiP	P11021	8.32E+07	9.73E+07	1.01E+08	1.08E+08
Endoplasmin	A0A7P0T917	4.65E+07	5.29E+07	5.39E+07	5.43E+07
Extracellular matrix protein 1	Q16610	3.04E+08	2.98E+08	3.43E+08	3.23E+08
Extracellular superoxide dismutase [Cu-Zn]	P08294	2.54E+07	3.25E+07	3.37E+07	1.57E+08
Fibrinogen alpha chain	P02671	5.79E+07	6.54E+07	9.07E+07	7.75E+07
Fibronectin	P02751	1.31E+09	1.66E+09	1.69E+09	1.45E+09
Fibulin-1	P23142	3.65E+09	3.67E+09	3.59E+09	3.32E+09
Fibulin-1	B1AHL2	5.33E+07	5.56E+07	5.64E+07	5.35E+07
Ficolin-3	O75636	4.77E+07	3.24E+07	5.09E+07	5.45E+07
GDH/6PGL endoplasmic bifunctional protein	O95479	4.83E+07	9.63E+07	5.66E+07	6.74E+07
Gelsolin	P06396	5.12E+08	5.35E+08	6.05E+08	4.78E+08
Haptoglobin	P00738	1.43E+11	1.51E+11	9.90E+10	1.06E+11
Haptoglobin-related protein	P00739	5.81E+08	6.17E+08	4.03E+08	3.57E+08
Hemoglobin subunit alpha	P69905	3.75E+09	6.15E+09	2.70E+09	3.81E+09
Hemoglobin subunit beta	P68871	5.75E+09	7.38E+09	3.29E+09	8.10E+09

Hemopexin	P02790	1.43E+09	1.46E+09	1.66E+09	1.69E+09
Heparin cofactor 2	P05546	5.11E+08	5.59E+08	6.43E+08	6.65E+08
Hepatocyte growth factor activator	Q04756	2.06E+09	2.17E+09	2.40E+09	2.50E+09
Histidine-rich glycoprotein	P04196	9.48E+08	8.76E+08	9.14E+08	9.55E+08
Histone H2A	A0A0U1RRH7	1.20E+08	1.59E+08	1.61E+08	1.43E+08
Hyaluronan-binding protein 2	Q14520	4.15E+07	3.99E+07	4.51E+07	3.95E+07
Hypoxia up-regulated protein 1	A0A087X054	2.88E+07	2.71E+07	2.38E+07	2.56E+07
Ig-like domain-containing protein (Fragment)	A0A0G2JRQ6	3.55E+08	3.74E+08	3.50E+08	3.84E+08
Ig-like domain-containing protein (Fragment)	A0A0J9YY99	5.84E+09	6.43E+09	5.28E+09	5.23E+09
Immunoglobulin heavy constant alpha 1	P01876	1.25E+11	1.10E+11	9.54E+10	9.42E+10
Immunoglobulin heavy constant alpha 2 (Fragment)	A0A0G2JMB2	4.69E+09	6.25E+09	4.61E+09	4.40E+09
Immunoglobulin heavy constant delta	P01880	2.03E+09	9.35E+08	1.54E+09	1.92E+09
Immunoglobulin heavy constant gamma 1	P01857	5.86E+10	5.64E+10	5.03E+10	4.68E+10
Immunoglobulin heavy constant gamma 2	P01859	2.66E+10	2.69E+10	2.55E+10	2.24E+10
Immunoglobulin heavy constant gamma 3	P01860	4.77E+10	4.83E+10	4.40E+10	3.94E+10
Immunoglobulin heavy constant gamma 4	P01861	7.38E+09	6.59E+09	7.13E+09	6.12E+09
Immunoglobulin heavy constant mu	P01871	2.96E+09	3.34E+09	2.87E+09	3.13E+09
Immunoglobulin heavy variable 1-18	A0A0C4DH31	9.77E+08	1.03E+09	9.33E+08	9.68E+08
Immunoglobulin heavy variable 1-2	P23083	2.96E+09	3.22E+09	2.76E+09	2.58E+09
Immunoglobulin heavy variable 1-24	A0A0C4DH33	9.69E+07	1.32E+08	6.01E+07	9.34E+07
Immunoglobulin heavy variable 1-45	A0A0A0MS14	1.56E+08	1.03E+08	1.31E+08	1.55E+08
Immunoglobulin heavy variable 1-46	P01743	1.45E+08	8.68E+07	9.54E+07	9.51E+07
Immunoglobulin heavy variable 1-69	P01742	2.04E+09	2.46E+09	2.51E+09	2.42E+09
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	A0A075B7D0	9.61E+08	1.26E+09	1.34E+09	9.44E+08
Immunoglobulin heavy variable 2-26	A0A0B4J1V2	8.72E+07	1.39E+08	9.80E+07	9.73E+07
Immunoglobulin heavy variable 2-70D	A0A0C4DH43	7.69E+08	7.29E+08	1.60E+09	8.49E+08
Immunoglobulin heavy variable 3-13	P01766	3.35E+08	2.91E+08	2.78E+08	3.38E+08
Immunoglobulin heavy variable 3-15	A0A0B4J1V0	8.22E+08	8.55E+08	9.92E+08	8.31E+08
Immunoglobulin heavy variable 3-43	A0A0B4J1X8	1.21E+08	1.96E+08	1.37E+08	1.87E+08
Immunoglobulin heavy variable 3-49	A0A0A0MS15	6.59E+08	6.99E+08	6.11E+08	5.28E+08
Immunoglobulin heavy variable 3-64D	A0A0J9YX35	3.35E+08	3.41E+08	3.10E+08	3.64E+08
Immunoglobulin heavy variable 3-7	P01780	7.24E+09	7.44E+09	7.43E+09	6.69E+09
Immunoglobulin heavy variable 3-72	A0A4W8ZXM2	7.68E+09	7.68E+09	8.71E+09	7.74E+09
Immunoglobulin heavy variable 3-73	A0A0B4J1V6	3.65E+08	4.39E+08	4.89E+08	3.58E+08
Immunoglobulin heavy variable 3-74	A0A0B4J1X5	8.30E+08	1.00E+09	1.15E+09	1.10E+09
Immunoglobulin heavy variable 3-9	P01782	9.40E+08	9.47E+08	7.49E+08	7.27E+08
Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Fragment)	A0A075B7B8	1.81E+09	2.02E+09	2.42E+09	2.39E+09
Immunoglobulin heavy variable 4-28	A0A0C4DH34	1.19E+09	1.33E+09	1.11E+09	1.40E+09
Immunoglobulin heavy variable 4-38-2	P0DP08	1.14E+09	1.36E+09	1.26E+09	1.43E+09
Immunoglobulin heavy variable 4-4	A0A075B6R2	9.05E+08	9.02E+08	8.41E+08	6.49E+08
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	A0A075B7B6	2.58E+08	2.73E+08	3.52E+08	3.22E+08
Immunoglobulin heavy variable 5-51	A0A0C4DH38	2.15E+09	2.16E+09	1.77E+09	1.73E+09
Immunoglobulin heavy variable 6-1	A0A0B4J1U7	1.50E+09	1.83E+09	3.17E+09	3.21E+09
Immunoglobulin J chain	P01591	3.06E+08	4.02E+08	3.67E+08	3.37E+08

Immunoglobulin kappa constant	P01834	4.50E+10	4.43E+10	3.55E+10	3.46E+10
Immunoglobulin kappa variable 1-17	P01599	1.62E+08	1.73E+08	1.83E+08	1.40E+08
Immunoglobulin kappa variable 1-33	A0A2Q2TTZ9	3.64E+09	3.32E+09	3.16E+09	2.90E+09
Immunoglobulin kappa variable 1-6	A0A0C4DH72	8.32E+07	9.14E+07	7.41E+07	8.73E+07
Immunoglobulin kappa variable 1-8	A0A0C4DH67	1.35E+08	1.27E+08	1.55E+08	1.41E+08
Immunoglobulin kappa variable 2-24	A0A0C4DH68	7.91E+08	8.85E+08	7.47E+08	1.06E+09
Immunoglobulin kappa variable 2-30	P06310	6.40E+08	8.55E+08	8.54E+08	7.76E+08
Immunoglobulin kappa variable 2-40	A0A087X0Q4	8.84E+09	9.24E+09	9.06E+09	8.11E+09
Immunoglobulin kappa variable 2D-28	A0A5H1ZRS2	5.87E+08	5.10E+08	4.64E+08	3.64E+08
Immunoglobulin kappa variable 2D-29	A0A5H1ZRS9	3.75E+08	3.52E+08	5.53E+08	3.52E+08
Immunoglobulin kappa variable 3-11	P04433	5.53E+09	5.09E+09	7.56E+09	5.62E+09
Immunoglobulin kappa variable 3-20	P01619	2.44E+09	2.52E+09	2.63E+09	2.41E+09
Immunoglobulin kappa variable 4-1	P06312	1.34E+09	1.29E+09	1.51E+09	1.47E+09
Immunoglobulin kappa variable 6-21	A0A0C4DH24	1.46E+08	1.22E+08	1.02E+08	1.18E+08
Immunoglobulin lambda constant 2	P0DOY2	6.25E+07	3.00E+07	1.20E+08	6.16E+07
Immunoglobulin lambda constant 3	P0DOY3	4.21E+10	3.77E+10	3.40E+10	3.37E+10
Immunoglobulin lambda constant 7 (Fragment)	A0A5H1ZRQ7	1.88E+08	1.65E+08	2.60E+08	1.73E+08
Immunoglobulin lambda variable 1-47	P01700	2.21E+09	1.70E+09	1.80E+09	1.67E+09
Immunoglobulin lambda variable 1-51	P01701	1.48E+08	1.70E+08	1.68E+08	1.81E+08
Immunoglobulin lambda variable 2-11	P01706	1.64E+08	1.81E+08	1.52E+08	1.58E+08
Immunoglobulin lambda variable 3-10	A0A075B6K4	2.32E+09	2.10E+09	2.50E+09	2.39E+09
Immunoglobulin lambda variable 3-16	A0A075B6K0	7.24E+08	7.94E+08	8.39E+08	7.09E+08
Immunoglobulin lambda variable 3-19	P01714	6.93E+08	7.17E+08	8.01E+08	8.14E+08
Immunoglobulin lambda variable 3-21	P80748	2.31E+08	1.75E+08	2.05E+08	1.69E+08
Immunoglobulin lambda variable 3-9	A0A075B6K5	2.09E+09	1.78E+09	2.08E+09	1.83E+09
Immunoglobulin lambda variable 4-69	A0A075B6H9	1.27E+08	1.12E+08	1.23E+08	1.02E+08
Immunoglobulin lambda variable 5-45 (Fragment)	A0A0G2JSC0	5.34E+07	4.51E+07	4.64E+07	4.49E+07
Immunoglobulin lambda variable 7-46	A0A075B6I9	4.69E+08	5.17E+08	5.51E+08	5.65E+08
Immunoglobulin lambda variable 8-61	A0A075B6I0	3.66E+08	7.06E+08	4.20E+08	5.14E+08
Immunoglobulin lambda variable 9-49	A0A0B4J1Y8	1.58E+08	1.12E+08	9.14E+07	1.24E+08
Immunoglobulin lambda-like polypeptide 5	B9A064	5.73E+09	5.62E+09	6.48E+09	5.94E+09
Insulin-like growth factor-binding protein 3	A6XND1	4.52E+07	4.04E+07	4.00E+07	6.16E+07
Insulin-like growth factor-binding protein complex acid labile subunit	P35858	2.79E+08	2.21E+08	2.54E+08	2.62E+08
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	2.15E+10	2.17E+10	2.48E+10	2.16E+10
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	4.56E+10	4.16E+10	4.42E+10	4.12E+10
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033	7.00E+09	9.79E+09	7.06E+09	6.19E+09
Intercellular adhesion molecule 1	P05362	6.08E+07	8.26E+07	7.21E+07	7.11E+07
Interleukin-1 receptor accessory protein	Q9NPH3	4.36E+07	5.74E+07	3.19E+07	4.78E+07
ITIH4 protein	B7ZKJ8	3.57E+09	3.77E+09	4.04E+09	4.06E+09
Kallistatin	P29622	3.75E+07	3.79E+07	3.86E+07	3.55E+07
Keratin, type I cytoskeletal 10	P13645	2.40E+08	5.53E+08	2.34E+08	2.66E+08
Keratin, type I cytoskeletal 9	P35527	1.62E+08	1.11E+08	7.78E+07	1.84E+08
Keratin, type II cytoskeletal 1	P04264	4.14E+08	4.43E+08	2.95E+08	4.31E+08
Kininogen-1	P01042	1.35E+10	1.45E+10	1.38E+10	1.64E+10
L-selectin	P14151	5.59E+08	4.80E+08	4.13E+08	3.54E+08

Low affinity immunoglobulin gamma Fc region receptor III-A	P08637	2.45E+08	2.93E+08	3.06E+08	2.91E+08
Lumican	P51884	2.02E+09	2.35E+09	1.82E+09	1.79E+09
Lymphatic vessel endothelial hyaluronic acid receptor 1	Q9Y5Y7	5.54E+07	7.20E+07	6.95E+07	7.33E+07
Macrophage colony-stimulating factor 1 receptor	P07333	7.79E+07	9.14E+07	6.70E+07	8.64E+07
Mannan-binding lectin serine protease 1	P48740	5.48E+08	5.95E+08	6.03E+08	7.43E+08
Mannan-binding lectin serine protease 2	O00187	1.04E+08	1.23E+08	1.11E+08	1.31E+08
Mast/stem cell growth factor receptor Kit	P10721	1.31E+10	1.37E+10	1.33E+10	1.16E+10
Matrix metalloproteinase-9	P14780	7.90E+07	8.79E+07	8.06E+07	8.53E+07
N-acetylmuramoyl-L-alanine amidase	Q96PD5	4.97E+07	5.00E+07	4.66E+07	6.75E+07
Phosphatidylcholine-sterol acyltransferase	P04180	4.13E+08	4.65E+08	4.54E+08	4.92E+08
Phosphatidylinositol-glycan-specific phospholipase D	P80108	3.42E+09	3.24E+09	3.92E+09	4.91E+09
Phospholipid transfer protein	P55058	1.14E+08	9.99E+07	1.28E+08	1.06E+08
Plasma kallikrein	P03952	1.27E+09	1.39E+09	1.34E+09	2.35E+09
Plasma protease C1 inhibitor	A0A7I2V2D2	4.00E+10	4.35E+10	3.90E+10	4.17E+10
Plasminogen	P00747	2.32E+08	2.67E+08	2.65E+08	2.81E+08
Platelet factor 4	P02776	9.61E+07	1.01E+08	8.15E+07	8.71E+07
Plexin domain-containing protein 2	Q6UX71	9.03E+07	1.01E+08	1.18E+08	1.31E+08
Pregnancy zone protein	P20742	3.57E+08	1.08E+08	7.03E+08	4.89E+08
Prenylcysteine oxidase 1	Q9UHG3	4.15E+08	4.39E+08	3.90E+08	3.73E+08
Probable non-functional immunoglobulin heavy variable 3-38	A0A0C4DH36	2.95E+08	2.65E+08	2.47E+08	3.01E+08
Properdin	P27918	3.18E+08	2.75E+08	4.73E+08	4.20E+08
Protein AMBP	P02760	1.24E+10	1.26E+10	1.34E+10	1.26E+10
Protein Z-dependent protease inhibitor	Q9UK55	3.63E+07	4.41E+07	4.36E+07	3.77E+07
Prothrombin	P00734	5.86E+09	4.40E+09	5.76E+09	5.62E+09
SAA2-SAA4	A0A096LPE2	5.05E+09	6.68E+09	4.33E+09	4.24E+09
Scavenger receptor cysteine-rich type 1 protein M130	F5GZZ9	1.05E+08	1.13E+08	1.85E+08	1.49E+08
Selenoprotein P	P49908	6.62E+08	7.29E+08	7.46E+08	9.52E+08
Serotransferrin	P02787	4.97E+09	5.39E+09	5.37E+09	5.33E+09
Serum amyloid P-component	P02743	1.19E+08	6.74E+07	1.04E+08	1.50E+08
Serum paraoxonase/arylesterase 1	P27169	1.12E+10	1.03E+10	7.71E+09	7.79E+09
Serum paraoxonase/lactonase 3	Q15166	2.91E+08	2.90E+08	2.04E+08	1.54E+08
Sulfhydryl oxidase 1	O00391	1.19E+08	1.37E+08	1.37E+08	1.59E+08
Tetranectin	E9PHK0	1.37E+07	1.28E+07	1.34E+07	1.15E+07
Transferrin receptor protein 1	P02786	7.50E+08	9.44E+08	7.40E+08	1.04E+09
Transforming growth factor-beta-induced protein ig-h3	Q15582	5.09E+07	5.80E+07	4.39E+07	5.13E+07
Transthyretin	A0A087WT59	1.56E+08	1.31E+08	1.63E+08	1.55E+08
Uncharacterized protein C16orf46	Q6P387	1.16E+09	1.34E+09	1.27E+09	1.10E+09
Vasorin	Q6EMK4	2.01E+08	1.79E+08	1.83E+08	1.89E+08
Vitamin D-binding protein	P02774	1.90E+08	1.93E+08	2.67E+08	2.55E+08
Vitamin K-dependent protein C	P04070	9.51E+06	1.12E+07	8.91E+06	9.66E+06
Vitamin K-dependent protein S	A0A3B3ISJ1	7.52E+08	7.94E+08	8.30E+08	9.14E+08
Vitamin K-dependent protein Z	P22891	8.90E+07	8.36E+07	1.15E+08	1.34E+08
Vitronectin	P04004	5.52E+09	4.86E+09	5.89E+09	6.32E+09
von Willebrand factor	P04275	1.87E+08	1.68E+08	2.04E+08	2.04E+08

Appendix 4C – All proteins detected on S-HDL-P

198 proteins were detected on S-HDL-P and are shown below.

Table 4.18 *Proteins detected on small HDL-P across groups.*

Protein names	Majority protein IDs	Con1/ Non-OB	T1DM	Con2/ OB	T2DM
	Uniprot	Mean LFQ Intensity			
72 kDa type IV collagenase	P08253	1.38E+07	1.43E+07	1.69E+07	1.23E+07
A-kinase anchor protein 9	A0A2R8Y590	1.67E+08	1.63E+08	1.48E+08	1.36E+08
Actin, cytoplasmic 1	P60709	2.80E+08	2.51E+08	3.05E+08	2.85E+08
Adipocyte plasma membrane-associated protein	Q9HDC9	4.41E+07	5.31E+07	3.79E+07	5.04E+07
Afamin	P43652	9.09E+09	7.54E+09	9.55E+09	9.54E+09
Albumin	P02768	2.98E+11	2.85E+11	2.58E+11	2.41E+11
Alpha-1-acid glycoprotein 1	P02763	4.29E+07	4.06E+07	4.19E+07	4.34E+07
Alpha-1-acid glycoprotein 2	P19652	1.26E+08	1.40E+08	9.93E+07	1.11E+08
Alpha-1-antichymotrypsin	P01011	1.28E+10	1.37E+10	1.18E+10	1.08E+10
Alpha-1-antitrypsin	P01009	8.25E+09	6.68E+09	7.12E+09	5.12E+09
Alpha-1B-glycoprotein	P04217	1.61E+10	1.88E+10	1.66E+10	1.62E+10
Alpha-2-antiplasmin	P08697	3.15E+09	3.51E+09	2.86E+09	3.30E+09
Alpha-2-HS-glycoprotein	P02765	1.00E+10	8.76E+09	9.27E+09	1.07E+10
Alpha-2-macroglobulin	P01023	4.74E+08	4.98E+08	3.83E+08	3.46E+08
Angiotensinogen	A0A7P0T9S6	8.38E+08	9.41E+08	9.52E+08	1.01E+09
Antithrombin-III	P01008	7.00E+08	6.53E+08	5.06E+08	4.67E+08
Apolipoprotein A-I	P02647	1.59E+11	1.51E+11	1.50E+11	1.57E+11
Apolipoprotein A-II	P02652	2.20E+10	1.89E+10	2.31E+10	2.25E+10
Apolipoprotein A-IV	P06727	4.64E+09	6.07E+09	4.51E+09	6.31E+09
Apolipoprotein B-100	P04114	1.52E+08	1.05E+08	1.42E+08	1.47E+08
Apolipoprotein C-I	K7ERI9	1.45E+09	1.46E+09	1.50E+09	1.85E+09
Apolipoprotein C-II	K7ER74	4.23E+08	3.11E+08	4.79E+08	6.79E+08
Apolipoprotein C-III	P02656	2.57E+09	2.16E+09	3.08E+09	3.97E+09
Apolipoprotein C-IV	P55056	1.01E+08	7.78E+07	9.21E+07	1.10E+08
Apolipoprotein D	P05090	3.92E+09	3.19E+09	3.33E+09	3.11E+09
Apolipoprotein E	P02649	1.35E+09	1.29E+09	1.49E+09	1.82E+09
Apolipoprotein L1	O14791	1.55E+08	1.00E+08	2.17E+08	2.99E+08
Apolipoprotein M	O95445	1.98E+09	2.06E+09	2.15E+09	2.24E+09
Beta-2-glycoprotein 1	P02749	1.46E+09	1.89E+09	1.73E+09	1.90E+09
Beta-Ala-His dipeptidase	Q96KN2	3.21E+08	3.09E+08	3.34E+08	3.49E+08
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	Q9H3R1	8.22E+08	1.01E+09	7.22E+08	9.02E+08
Biotinidase	P43251	1.58E+08	1.66E+08	1.74E+08	1.78E+08
C3/C5 convertase	B4E1Z4	3.04E+10	3.38E+10	3.47E+10	4.01E+10
C4b-binding protein alpha chain	P04003	1.17E+08	1.51E+08	1.16E+08	1.31E+08
CD5 antigen-like	O43866	4.28E+07	4.88E+07	4.55E+07	4.75E+07
Ceruloplasmin	P00450	5.68E+10	6.67E+10	5.94E+10	5.65E+10
Clusterin	P10909	1.40E+10	1.43E+10	1.47E+10	1.62E+10
Coagulation factor IX	P00740	5.47E+08	6.03E+08	5.88E+08	7.63E+08

Coagulation factor V	P12259	1.22E+08	1.17E+08	1.13E+08	1.29E+08
Coagulation factor X	P00742	9.21E+08	9.11E+08	9.43E+08	9.73E+08
Coagulation factor XII	P00748	2.13E+08	2.06E+08	2.38E+08	2.35E+08
Coagulation factor XIII B chain	P05160	1.30E+09	1.28E+09	1.36E+09	1.11E+09
Complement C1r subcomponent-like protein	Q9NZP8	2.71E+08	2.88E+08	2.66E+08	3.14E+08
Complement C1s subcomponent	P09871	1.11E+09	9.61E+08	1.17E+09	1.09E+09
Complement C2	P06681	3.56E+08	4.37E+08	3.69E+08	4.10E+08
Complement C3	P01024	3.31E+11	3.26E+11	3.73E+11	3.89E+11
Complement C4-A	P0C0L4	7.37E+08	7.62E+08	8.64E+08	8.06E+08
Complement C4-B	P0C0L5	2.91E+10	3.10E+10	3.40E+10	3.08E+10
Complement C5	P01031	9.58E+09	1.05E+10	1.16E+10	1.21E+10
Complement component C6	P13671	2.25E+09	2.33E+09	2.64E+09	2.44E+09
Complement component C7	P10643	2.45E+09	2.73E+09	2.22E+09	2.63E+09
Complement component C8 alpha chain	P07357	1.08E+09	1.36E+09	1.16E+09	1.35E+09
Complement component C8 beta chain	P07358	1.29E+09	1.59E+09	1.48E+09	1.62E+09
Complement component C8 gamma chain	P07360	9.14E+08	1.04E+09	9.98E+08	1.08E+09
Complement component C9	P02748	2.44E+09	2.99E+09	2.79E+09	2.73E+09
Complement factor H	P08603	1.07E+09	1.11E+09	1.14E+09	1.21E+09
Complement factor H-related protein 1	Q03591	2.45E+09	2.69E+09	2.53E+09	2.58E+09
Complement factor H-related protein 2	P36980	7.49E+08	6.94E+08	6.52E+08	7.45E+08
Complement factor I	G3XAM2	1.41E+09	1.49E+09	1.60E+09	1.71E+09
Complement subcomponent C1r	A0A3B3ISR2	1.22E+08	1.50E+08	1.49E+08	2.22E+08
Cysteine-rich secretory protein 3	P54108	5.65E+07	5.25E+07	4.50E+07	4.14E+07
EGF-containing fibulin-like extracellular matrix protein 1	Q12805	1.11E+08	1.19E+08	1.16E+08	1.04E+08
Endoplasmic reticulum chaperone BiP	P11021	4.35E+07	4.89E+07	4.51E+07	5.82E+07
Extracellular matrix protein 1	Q16610	4.10E+08	3.93E+08	3.72E+08	4.01E+08
Fibrinogen alpha chain	P02671	3.32E+07	3.13E+07	4.17E+07	3.92E+07
Fibronectin	P02751	5.38E+07	5.33E+07	3.64E+07	4.68E+07
Fibulin-1	P23142	1.50E+07	9.39E+06	1.02E+07	1.13E+07
Fructose-bisphosphate aldolase	A0A3B3IS80	2.48E+07	2.76E+07	4.28E+07	4.90E+07
Gelsolin	P06396	1.10E+09	1.02E+09	1.04E+09	9.47E+08
Glutathione peroxidase	A0A087X1J7	1.62E+08	1.73E+08	1.75E+08	1.84E+08
Haptoglobin	P00738	6.54E+09	1.06E+10	6.73E+09	6.36E+09
Haptoglobin-related protein	P00739	4.48E+07	5.93E+07	3.95E+07	3.60E+07
Hemoglobin subunit alpha	P69905	9.11E+07	1.05E+08	7.96E+07	1.52E+08
Hemoglobin subunit beta	P68871	3.04E+07	5.79E+07	5.04E+07	1.13E+08
Hemopexin	P02790	4.70E+10	4.47E+10	4.38E+10	4.64E+10
Heparin cofactor 2	P05546	6.13E+09	7.11E+09	7.53E+09	8.25E+09
Hepatocyte growth factor activator	Q04756	1.39E+08	1.32E+08	1.49E+08	1.47E+08
Histidine-rich glycoprotein	P04196	3.49E+09	3.37E+09	3.37E+09	3.88E+09
Hyaluronan-binding protein 2	Q14520	6.44E+07	6.94E+07	6.28E+07	7.80E+07
Ig-like domain-containing protein (Fragment)	A0A0G2JRQ6	3.93E+08	4.63E+08	3.46E+08	4.50E+08
Ig-like domain-containing protein (Fragment)	A0A0J9YY99	6.85E+09	7.22E+09	7.57E+09	6.39E+09
Immunoglobulin heavy constant alpha 1	P01876	1.09E+10	1.03E+10	9.18E+09	9.04E+09
Immunoglobulin heavy constant alpha 2 (Fragment)	A0A0G2JMB2	3.83E+08	4.06E+08	3.93E+08	3.11E+08

Immunoglobulin heavy constant gamma 1	P01857	1.03E+11	9.55E+10	9.64E+10	7.84E+10
Immunoglobulin heavy constant gamma 2	P01859	4.55E+10	4.46E+10	4.54E+10	4.33E+10
Immunoglobulin heavy constant gamma 3	P01860	2.29E+10	2.29E+10	2.13E+10	2.13E+10
Immunoglobulin heavy constant gamma 4	P01861	9.21E+09	8.06E+09	8.17E+09	7.37E+09
Immunoglobulin heavy constant mu	P01871	6.25E+08	6.22E+08	5.62E+08	6.07E+08
Immunoglobulin heavy variable 1-18	A0A0C4DH31	4.70E+08	4.45E+08	5.28E+08	3.94E+08
Immunoglobulin heavy variable 1-2	P23083	1.99E+09	1.82E+09	2.00E+09	1.69E+09
Immunoglobulin heavy variable 1-24	A0A0C4DH33	6.70E+07	8.46E+07	6.33E+07	5.84E+07
Immunoglobulin heavy variable 1-46	P01743	1.80E+08	1.45E+08	1.12E+08	1.36E+08
Immunoglobulin heavy variable 1-69	P01742	2.56E+09	2.46E+09	2.45E+09	2.40E+09
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	A0A075B7D0	1.42E+09	1.86E+09	1.64E+09	1.07E+09
Immunoglobulin heavy variable 2-26	A0A0B4J1V2	2.88E+08	3.54E+08	2.36E+08	2.29E+08
Immunoglobulin heavy variable 2-70D	A0A0C4DH43	8.73E+08	6.86E+08	6.66E+08	5.62E+08
Immunoglobulin heavy variable 3-13	P01766	5.09E+08	4.29E+08	4.73E+08	3.56E+08
Immunoglobulin heavy variable 3-15	A0A0B4J1V0	8.28E+08	8.35E+08	8.75E+08	7.55E+08
Immunoglobulin heavy variable 3-43	A0A0B4J1X8	1.85E+08	1.76E+08	1.31E+08	1.13E+08
Immunoglobulin heavy variable 3-49	A0A0A0MS15	7.91E+08	8.28E+08	9.42E+08	8.07E+08
Immunoglobulin heavy variable 3-7	P01780	9.99E+09	1.11E+10	1.16E+10	1.03E+10
Immunoglobulin heavy variable 3-72	A0A4W8ZXM2	1.09E+10	1.11E+10	1.25E+10	1.01E+10
Immunoglobulin heavy variable 3-73	A0A0B4J1V6	5.23E+08	5.10E+08	5.69E+08	4.57E+08
Immunoglobulin heavy variable 3-74	A0A0B4J1X5	1.27E+09	1.52E+09	1.59E+09	1.32E+09
Immunoglobulin heavy variable 3-9	P01782	2.17E+09	1.77E+09	1.53E+09	1.59E+09
Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Fragment)	A0A075B7B8	2.39E+09	2.58E+09	2.32E+09	1.25E+09
Immunoglobulin heavy variable 4-28	A0A0C4DH34	1.87E+09	1.69E+09	1.78E+09	1.43E+09
Immunoglobulin heavy variable 4-30-2	A0A087WSY4	2.47E+08	2.89E+08	2.48E+08	2.60E+08
Immunoglobulin heavy variable 4-38-2	P0DP08	3.17E+09	3.70E+09	3.31E+09	2.81E+09
Immunoglobulin heavy variable 4-4	A0A075B6R2	6.87E+08	8.81E+08	7.42E+08	7.08E+08
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	A0A075B7B6	3.56E+08	4.83E+08	4.57E+08	3.20E+08
Immunoglobulin heavy variable 5-51	A0A0C4DH38	3.04E+09	3.03E+09	3.03E+09	2.60E+09
Immunoglobulin heavy variable 6-1	A0A0B4J1U7	1.39E+09	1.29E+09	1.20E+09	1.26E+09
Immunoglobulin J chain	P01591	4.39E+07	5.56E+07	5.15E+07	5.11E+07
Immunoglobulin kappa constant	P01834	4.81E+10	4.30E+10	4.52E+10	3.81E+10
Immunoglobulin kappa variable 1-17	P01599	3.77E+08	4.23E+08	4.44E+08	3.89E+08
Immunoglobulin kappa variable 1-33	A0A2Q2TTZ9	5.10E+09	5.25E+09	4.67E+09	4.21E+09
Immunoglobulin kappa variable 1-5	P01602	1.68E+09	1.86E+09	1.85E+09	1.59E+09
Immunoglobulin kappa variable 1-6	A0A0C4DH72	1.92E+08	1.51E+08	1.53E+08	1.24E+08
Immunoglobulin kappa variable 1-8	A0A0C4DH67	2.45E+08	2.36E+08	2.34E+08	1.98E+08
Immunoglobulin kappa variable 1D-39	P04432	6.63E+07	5.54E+07	7.09E+07	5.62E+07
Immunoglobulin kappa variable 2-24	A0A0C4DH68	1.75E+09	1.48E+09	1.52E+09	1.17E+09
Immunoglobulin kappa variable 2-30	P06310	1.15E+09	1.10E+09	1.31E+09	1.01E+09
Immunoglobulin kappa variable 2-40	A0A087X0Q4	8.13E+09	7.22E+09	8.03E+09	6.23E+09
Immunoglobulin kappa variable 2D-28	A0A5H1ZRS2	1.24E+09	1.01E+09	1.09E+09	7.43E+08
Immunoglobulin kappa variable 2D-29	A0A5H1ZRS9	6.26E+08	7.02E+08	9.91E+08	6.09E+08
Immunoglobulin kappa variable 3-11	P04433	5.54E+09	4.84E+09	4.68E+09	4.54E+09

Immunoglobulin kappa variable 3-20	P01619	6.62E+09	6.44E+09	7.06E+09	5.66E+09
Immunoglobulin kappa variable 3D-15	A0A087WSY6	6.46E+08	6.65E+08	6.60E+08	5.49E+08
Immunoglobulin kappa variable 4-1	P06312	2.90E+09	3.12E+09	3.11E+09	2.62E+09
Immunoglobulin kappa variable 6-21	A0A0C4DH24	4.19E+08	5.15E+08	3.43E+08	3.49E+08
Immunoglobulin lambda constant 2	P0DOY2	1.67E+07	1.09E+07	3.82E+07	1.07E+07
Immunoglobulin lambda constant 3	P0DOY3	2.73E+10	2.42E+10	2.61E+10	2.30E+10
Immunoglobulin lambda constant 7 (Fragment)	A0A5H1ZRQ7	1.32E+08	1.61E+08	1.27E+08	1.15E+08
Immunoglobulin lambda variable 1-40	P01703	2.02E+08	1.74E+08	1.70E+08	1.49E+08
Immunoglobulin lambda variable 1-47	P01700	1.85E+09	2.31E+09	2.05E+09	2.15E+09
Immunoglobulin lambda variable 1-51	P01701	2.77E+08	3.16E+08	2.51E+08	2.13E+08
Immunoglobulin lambda variable 2-11	P01706	3.15E+08	3.16E+08	2.55E+08	2.77E+08
Immunoglobulin lambda variable 2-14	P01704	3.76E+08	2.52E+08	2.28E+08	2.39E+08
Immunoglobulin lambda variable 2-18	A0A075B6J9	1.79E+08	1.92E+08	1.28E+08	1.32E+08
Immunoglobulin lambda variable 3-10	A0A075B6K4	1.20E+09	1.19E+09	1.23E+09	1.05E+09
Immunoglobulin lambda variable 3-16	A0A075B6K0	5.61E+08	5.08E+08	4.96E+08	4.02E+08
Immunoglobulin lambda variable 3-19	P01714	1.34E+09	1.26E+09	9.30E+08	1.01E+09
Immunoglobulin lambda variable 3-21	P80748	1.36E+08	1.42E+08	1.43E+08	1.26E+08
Immunoglobulin lambda variable 3-9	A0A075B6K5	1.40E+09	1.35E+09	1.14E+09	1.12E+09
Immunoglobulin lambda variable 4-60	A0A075B6I1	3.45E+07	3.56E+07	3.42E+07	3.25E+07
Immunoglobulin lambda variable 4-69	A0A075B6H9	2.46E+08	2.35E+08	2.19E+08	2.28E+08
Immunoglobulin lambda variable 5-45 (Fragment)	A0A0G2JSC0	5.58E+07	6.13E+07	4.48E+07	5.03E+07
Immunoglobulin lambda variable 7-46	A0A075B6I9	7.64E+08	8.02E+08	6.35E+08	6.50E+08
Immunoglobulin lambda variable 8-61	A0A075B6I0	6.14E+08	7.31E+08	5.41E+08	5.60E+08
Immunoglobulin lambda variable 9-49	A0A0B4J1Y8	8.69E+07	1.69E+08	1.11E+08	1.26E+08
Immunoglobulin lambda-like polypeptide 1	P15814	1.44E+08	1.21E+08	1.30E+08	9.19E+07
Immunoglobulin lambda-like polypeptide 5	B9A064	4.35E+09	4.31E+09	4.28E+09	3.87E+09
Insulin-like growth factor-binding protein 3	A6XND1	5.50E+08	4.16E+08	5.26E+08	5.85E+08
Insulin-like growth factor-binding protein complex acid labile subunit	P35858	2.95E+09	2.61E+09	2.84E+09	2.75E+09
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	5.98E+08	7.56E+08	5.09E+08	4.90E+08
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	7.81E+08	8.19E+08	6.31E+08	5.47E+08
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033	7.35E+07	1.12E+08	6.94E+07	6.45E+07
ITIH4 protein	B7ZKJ8	2.11E+10	2.17E+10	2.16E+10	2.23E+10
Kallistatin	P29622	3.04E+07	3.50E+07	3.34E+07	3.26E+07
Keratin, type I cytoskeletal 10	P13645	5.96E+07	6.52E+07	4.59E+07	6.37E+07
Keratin, type I cytoskeletal 9	P35527	9.65E+07	6.04E+07	7.47E+07	1.85E+08
Keratin, type II cytoskeletal 1	P04264	2.16E+08	1.08E+08	1.45E+08	1.75E+08
Kininogen-1	P01042	1.89E+10	2.20E+10	2.17E+10	2.39E+10
L-lactate dehydrogenase B chain	P07195	4.45E+07	5.09E+07	5.46E+07	5.55E+07
L-selectin	P14151	1.56E+08	1.38E+08	1.26E+08	1.25E+08
Low affinity immunoglobulin gamma Fc region receptor III-A	P08637	4.62E+07	3.22E+07	3.79E+07	3.99E+07
Lumican	P51884	2.93E+09	3.33E+09	2.95E+09	2.94E+09
N-acetylmuramoyl-L-alanine amidase	Q96PD5	1.31E+09	1.23E+09	1.25E+09	1.20E+09
Phosphatidylcholine-sterol acyltransferase	P04180	6.66E+07	6.01E+07	6.75E+07	6.73E+07
Phosphatidylinositol-glycan-specific phospholipase D	P80108	1.08E+08	1.53E+08	1.67E+08	1.38E+08
Plasma kallikrein	P03952	7.83E+08	8.54E+08	9.14E+08	1.32E+09

Plasma protease C1 inhibitor	A0A7I2V2D2	2.68E+08	2.55E+08	3.20E+08	3.18E+08
Plasminogen	P00747	3.84E+08	4.05E+08	4.54E+08	5.16E+08
Platelet factor 4	P02776	6.96E+07	7.75E+07	6.62E+07	7.85E+07
Probable non-functional immunoglobulin heavy variable 3-35	A0A0C4DH35	3.46E+08	4.36E+08	3.88E+08	3.79E+08
Probable non-functional immunoglobulin heavy variable 3-38	A0A0C4DH36	2.66E+08	2.43E+08	2.42E+08	1.87E+08
Protein AMBP	P02760	8.08E+08	8.31E+08	7.66E+08	7.50E+08
Protein Z-dependent protease inhibitor	Q9UK55	4.29E+08	4.12E+08	4.73E+08	4.81E+08
Prothrombin	P00734	1.07E+10	1.22E+10	8.76E+09	1.07E+10
SAA2-SAA4	A0A096LPE2	4.95E+09	4.71E+09	5.33E+09	5.91E+09
Selenoprotein P	P49908	2.85E+08	2.63E+08	2.77E+08	3.64E+08
Serotransferrin	P02787	2.21E+10	2.24E+10	2.07E+10	2.24E+10
Serum amyloid P-component	P02743	1.43E+09	1.27E+09	1.69E+09	1.81E+09
Serum paraoxonase/arylesterase 1	P27169	3.35E+09	2.82E+09	3.22E+09	3.32E+09
Sex hormone-binding globulin	I3L145	3.58E+08	4.39E+08	2.45E+08	1.95E+08
SPARC	P09486	2.98E+08	3.13E+08	3.28E+08	2.87E+08
Sulfhydryl oxidase 1	O00391	1.56E+08	1.48E+08	1.50E+08	1.58E+08
Tetranectin	E9PHK0	2.53E+09	2.10E+09	2.53E+09	2.34E+09
Thyroxine-binding globulin	P05543	3.40E+07	2.93E+07	3.89E+07	3.22E+07
Transferrin receptor protein 1	P02786	4.00E+07	3.61E+07	3.18E+07	4.14E+07
Transforming growth factor-beta-induced protein ig-h3	Q15582	2.34E+08	2.27E+08	2.53E+08	2.47E+08
Transthyretin	A0A087WT59	8.30E+07	4.55E+07	6.85E+07	4.95E+07
Vitamin D-binding protein	P02774	3.51E+08	3.36E+08	3.67E+08	3.57E+08
Vitamin K-dependent protein C	P04070	3.65E+08	3.55E+08	3.19E+08	3.79E+08
Vitamin K-dependent protein S	A0A3B3ISJ1	5.88E+08	6.48E+08	6.40E+08	6.54E+08
Vitamin K-dependent protein Z	P22891	5.34E+07	5.64E+07	5.52E+07	4.37E+07
Vitronectin	P04004	8.37E+09	8.47E+09	9.33E+09	1.04E+10

Appendix 4D – Modulation of the proteomic composition of L-HDL-P

I. Modulation of the proteomic composition of L-HDL-P in T1DM

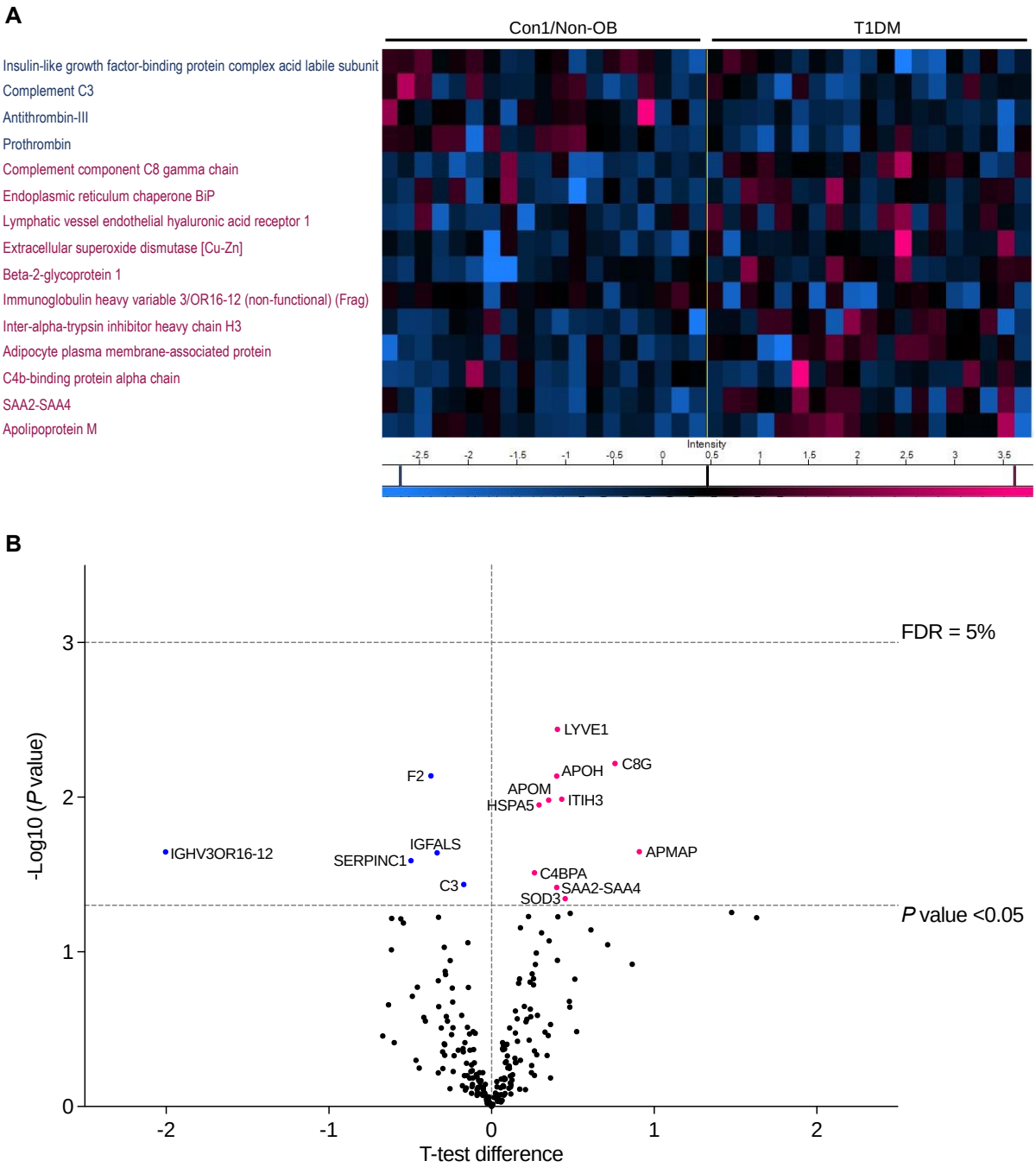

Figure 4.10 *Significantly changed proteins on large HDL-P between people with T1DM and matched controls.*

HDL was isolated via FPLC from people with and without T1DM (*n*=20 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between people with and without T1DM were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between controls and people with T1DM. Dots in pink indicate proteins increased in T1DM. Dots in blue indicate proteins decreased in T1DM. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

II. Modulation of the proteomic composition of L-HDL-P in T2DM

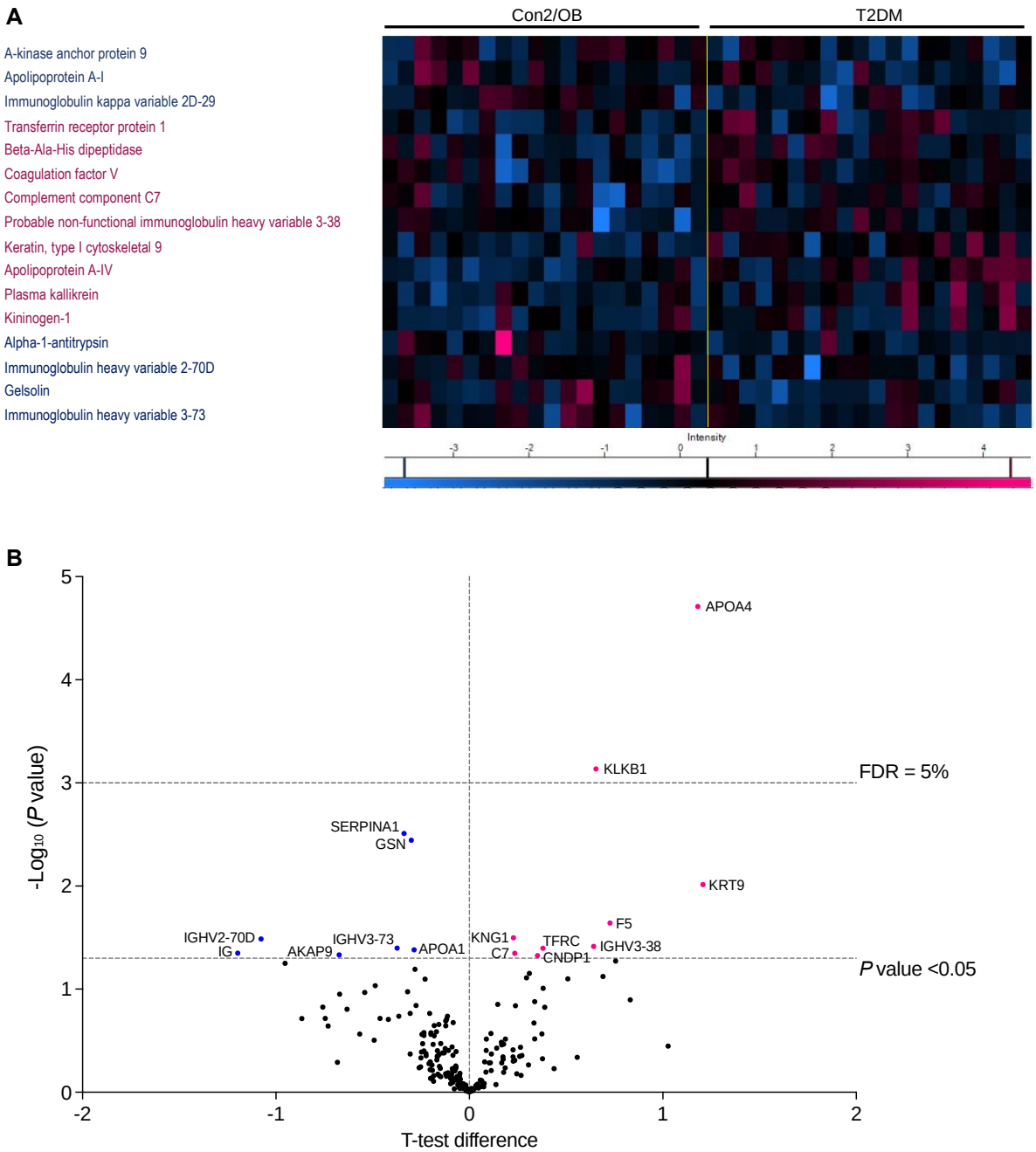

Figure 4.11 *Significantly changed proteins on large HDL-P between people with T2DM and matched controls.*
HDL was isolated via FPLC from people with and without T2DM (*n*=20 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between people with and without T2DM were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between controls and people with T2DM. Dots in pink indicate proteins increased in T2DM. Dots in blue indicate proteins decreased in T2DM. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

III. Modulation of the proteomic composition of L-HDL-P in controls with obesity relative to people without obesity

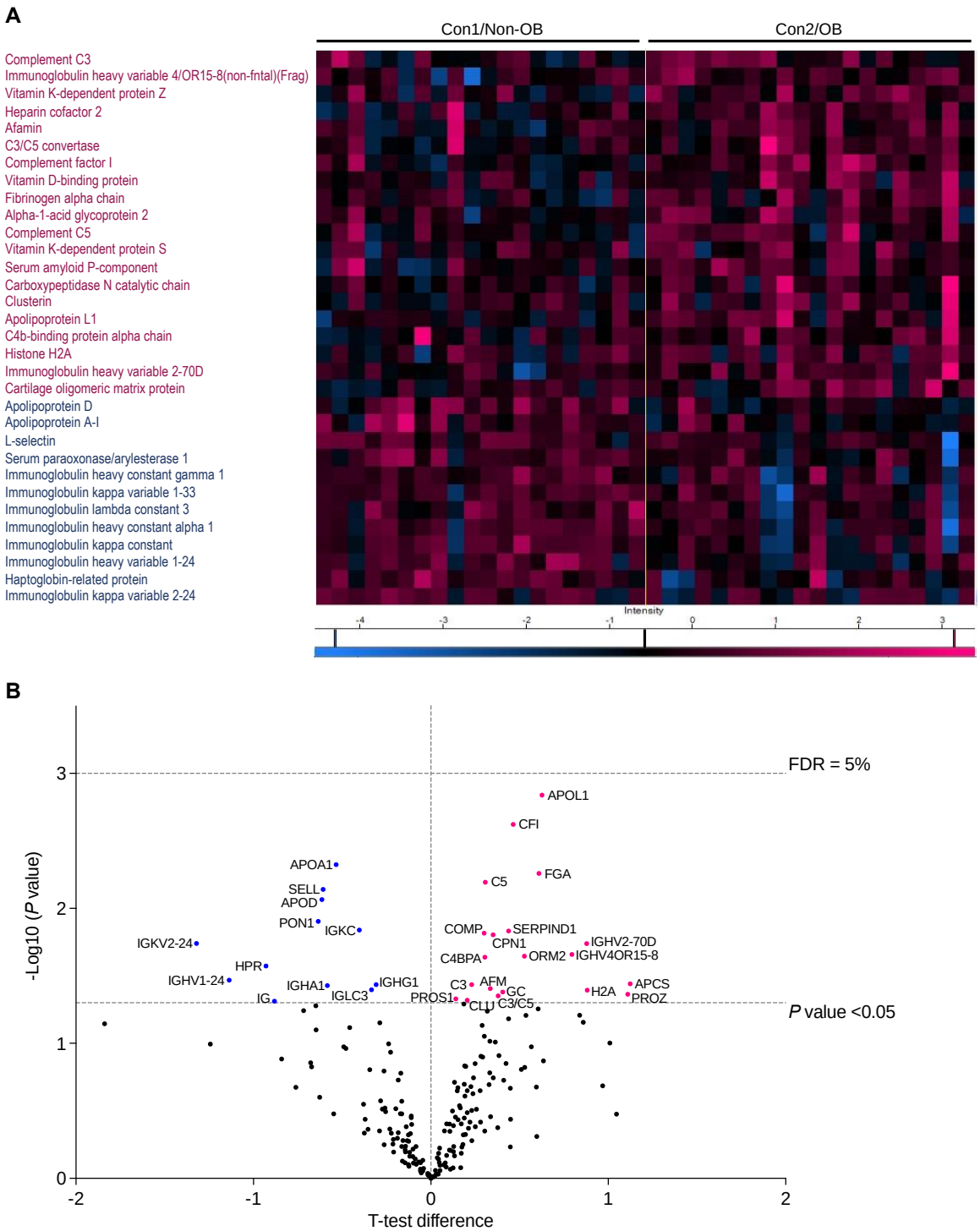

Figure 4.12 *Significantly changed proteins on large HDL-P between people with and without obesity.*

HDL was isolated via FPLC from people with and without obesity (*n*=20 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between people with and without obesity were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between people with and without obesity. Dots in pink indicate proteins increased in obesity. Dots in blue indicate proteins decreased in obesity. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

IV. Modulation of the proteome composition of L-HDL-P in the combined high BMI (OB&T2DM) group relative to non-obese group

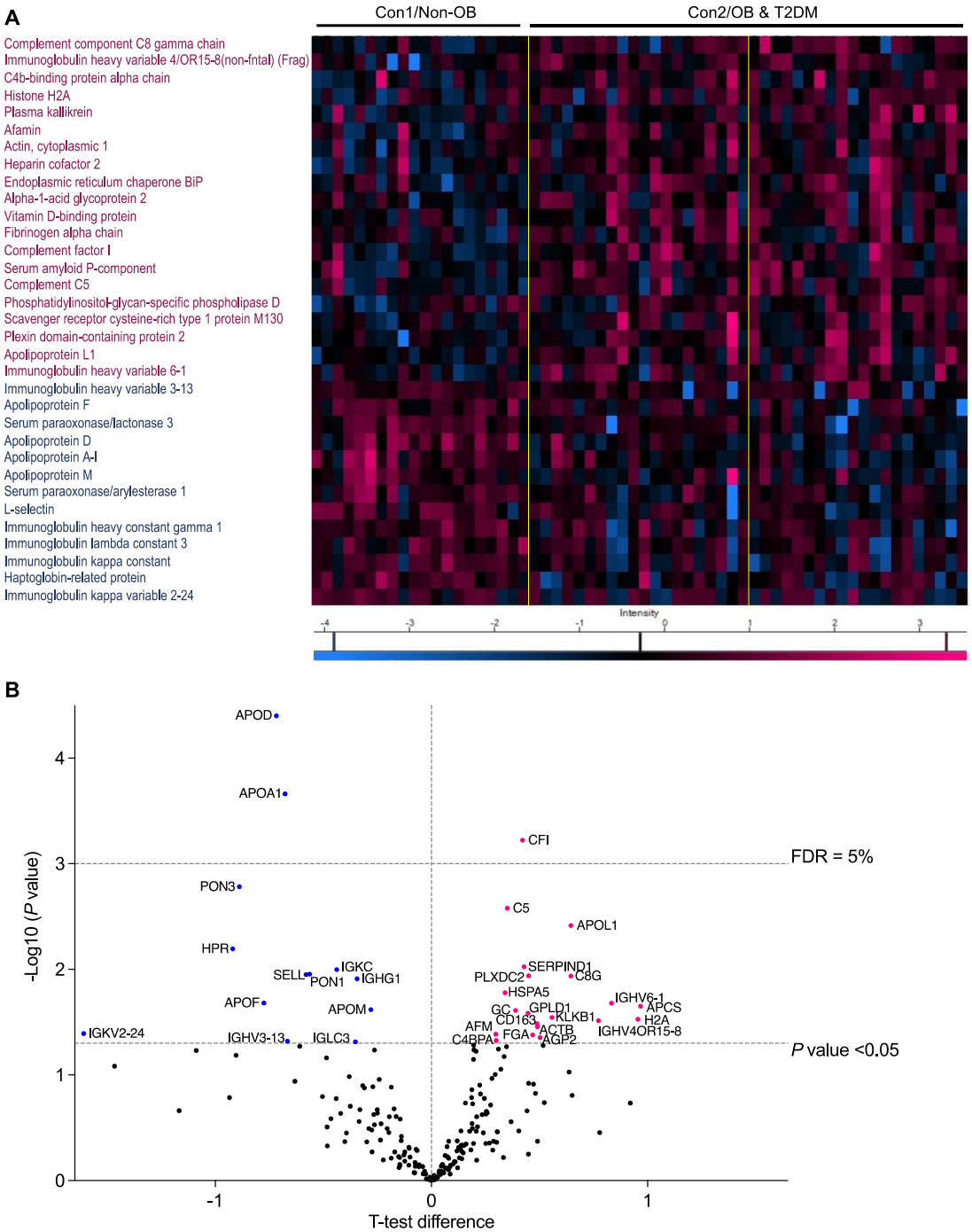

Figure 4.13 *Significantly changed proteins on large HDL-P between non-obese group and people with high BMI (combined obesity group).*
HDL was isolated via FPLC from people with and without obesity&T2DM (combined high BMI groups); non-obese (*n*=20) vs. OB&T2DM combined group (*n*=40). Differences in HDL protein levels on large HDL-P (fractions 36-38) between from people with and without obesity&T2DM (combined high BMI groups) were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between people with and without obesity&T2DM (combined high BMI groups). Dots in pink indicate proteins increased in OB&T2DM combined. Dots in blue indicate proteins decreased in OB&T2DM combined. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

Appendix 4E – Modulation of the proteomic composition of S-HDL-P

I. *Modulation of the proteomic composition of S-HDL-P in T1DM*

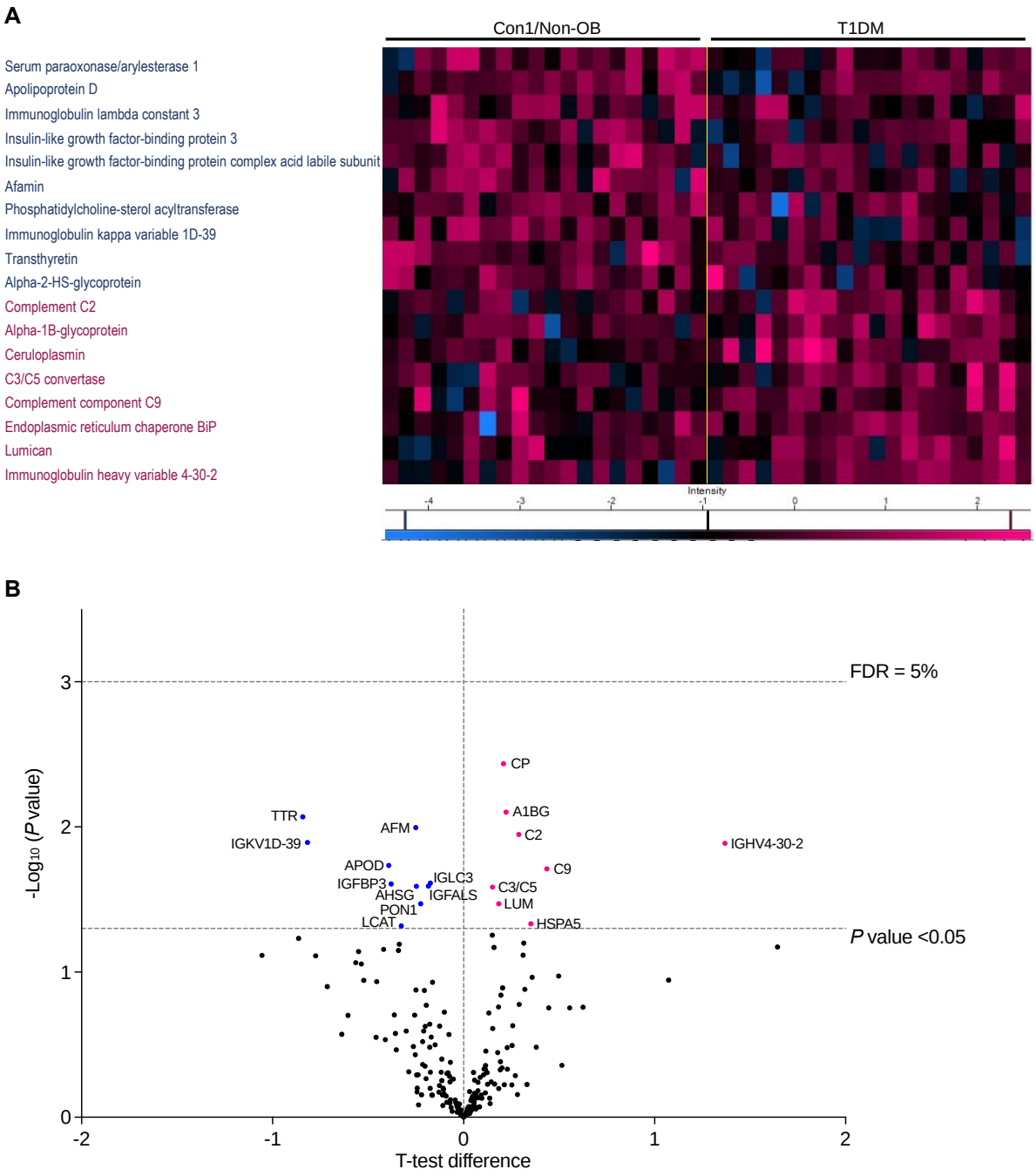

Figure 4.14 *Significantly changed proteins on small HDL-P between people with T1DM and matched controls.*

HDL was isolated via FPLC from people with and without T1DM (*n*=20 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between people with and without T1DM were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between controls and people with T1DM. Dots in pink indicate proteins increased in T1DM. Dots in blue indicate proteins decreased in T1DM. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

II. Modulation of the proteomic composition of S-HDL-P in T2DM

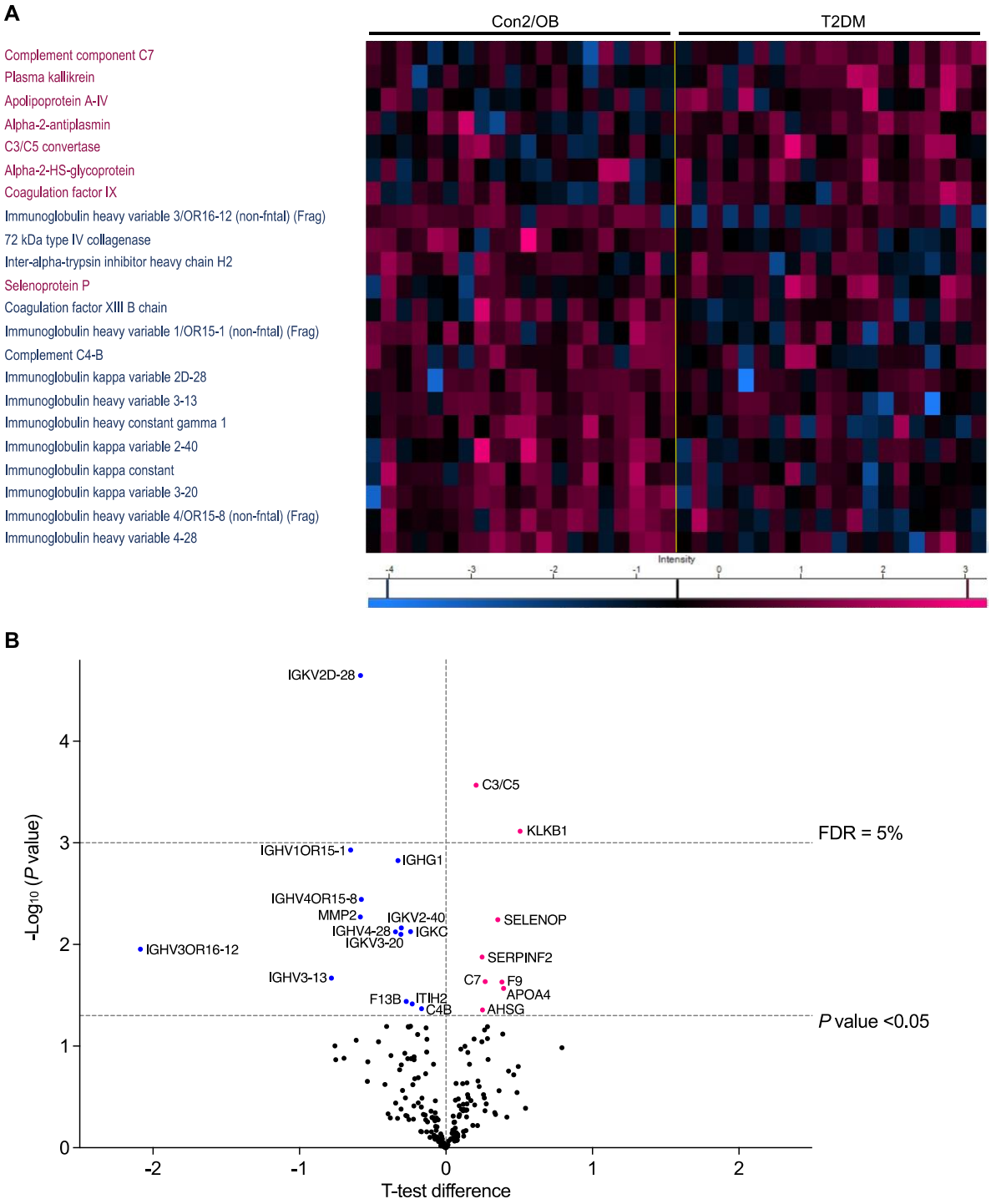

Figure 4.15 *Significantly changed proteins on small HDL-P between people with T2DM and matched controls.*

HDL was isolated via FPLC from people with and without T2DM (*n*=20 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between people with and without T2DM were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between controls and people with T2DM. Dots in pink indicate proteins increased in T2DM. Dots in blue indicate proteins that decreased in T2DM. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

III. Modulation of the proteomic composition of S-HDL-P in controls with obesity relative to people without obesity

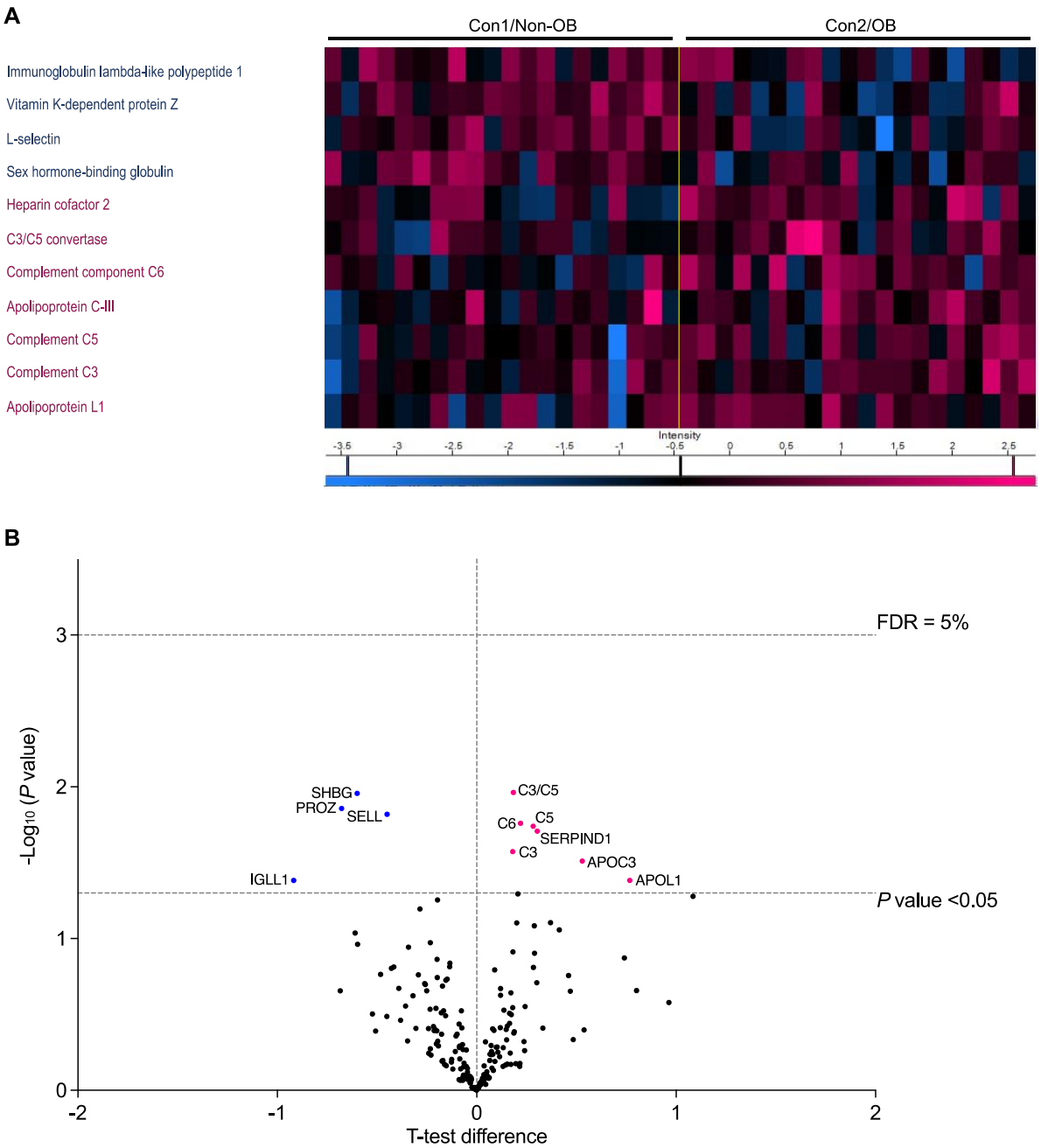

Figure 4.16 *Significantly changed proteins on small HDL-P between people with and without obesity.*
HDL was isolated via FPLC from people with and without obesity (*n*=20 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between people with and without obesity were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between people with and without obesity. Dots in pink indicate proteins increased in obesity. Dots in blue indicate proteins decreased in obesity. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a P value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

IV. Modulation of the proteomic composition of S-HDL-P in the combined high BMI group (OB&T2DM) relative to non-obese group

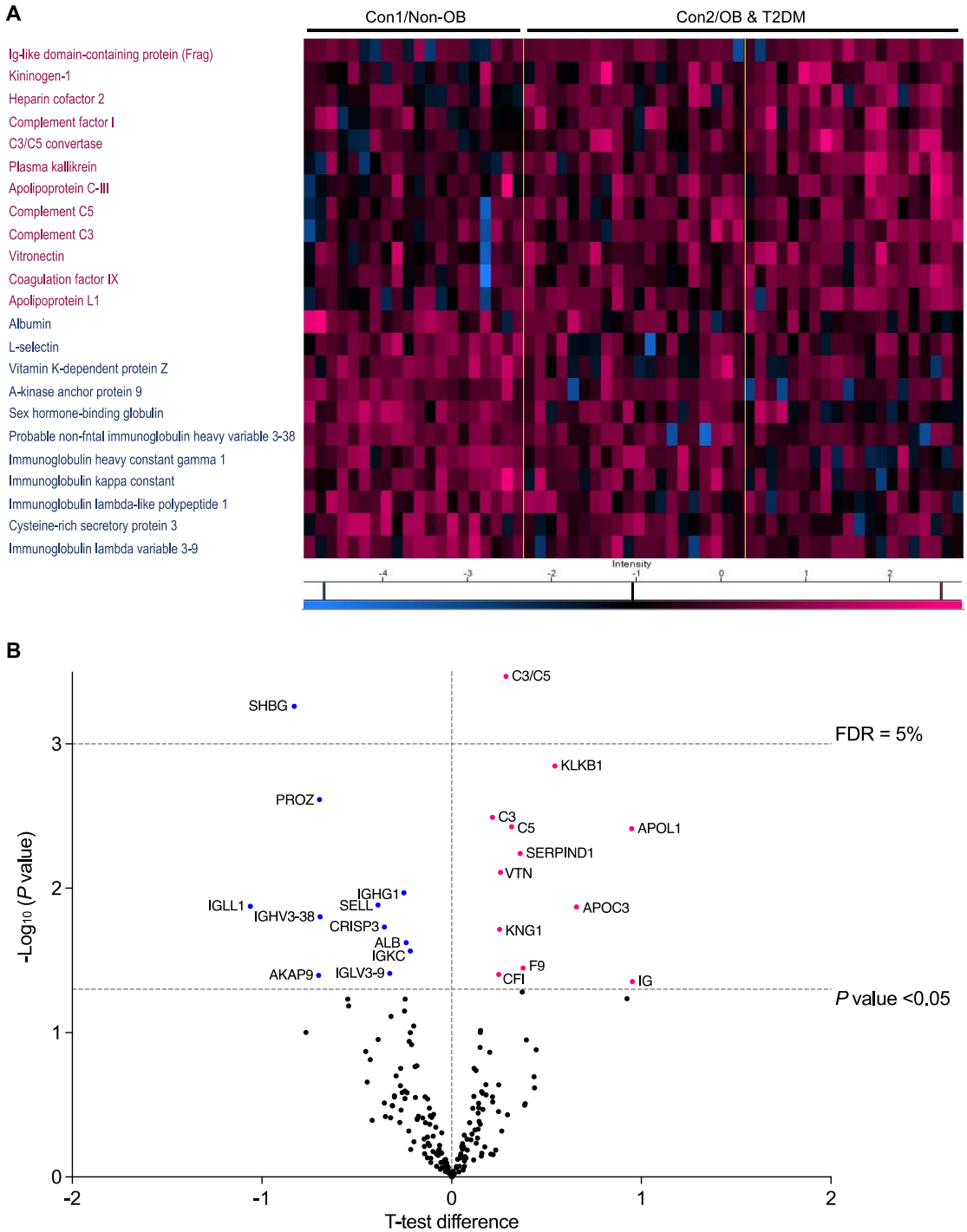

Figure 4.17 *Significantly changed proteins on small HDL-P between non-obese group and people with high BMI (Con2 and T2DM groups combined).*
HDL was isolated via FPLC from people with and without obesity&T2DM (combined high BMI groups); non-obese (*n*=20) vs. OB&T2DM combined group(*n*=40). Differences in HDL protein levels on small HDL-P (fractions 40-42) between people with and without obesity&T2DM (combined high BMI groups) were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (p<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between people with and without obesity&T2DM (combined high BMI groups). Dots in pink indicate proteins increased in OB&T2DM combined. Dots in blue indicate proteins decreased in OB&T2DM combined. Horizontal dotted lines indicate statistical thresholds for a FDR of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison.

Chapter 5

Sex-specific differences in HDL function and composition in people with and without metabolic disease.

Rachel Byrne, Eugene Dillon, Seán Curley, Mohamed Ahmed, Khalid Ahmed, Anne McGowan, Anjuli Gunness, Isolda Frizelle, Agnieszka Pazderska, Andrew Hogan, Donal O'Shea, James Gibney, and Fiona McGillicuddy.

Abstract

Introduction: Cardiovascular disease (CVD) pathophysiology has been predominantly researched in men, with less understood about disease pathogenesis in women. The protective effect of female sex on CVD risk is negated in the setting of diabetes but the underpinning mechanisms are poorly understood. Within this study, we investigated sex-specific differences in HDL-particle (HDL-P) size, cholesterol efflux capacity (CEC) and proteomic composition in men versus women with and without T1DM or T2DM.

Methods: Women and men with T1DM or T2DM, were age- and BMI-matched to control subjects (*n*=20 per group per sex). Total, ABCA1-dependent, and ABCA1-independent efflux were determined after 4h incubation of PEG-supernatants with ^{3}H-cholesterol labelled J774 macrophages treated ± cAMP *ex vivo*. Serum samples were sent for NMR lipoprotein analysis to LabCorp (LipoScience, Inc), NC, USA. HDL protein composition was investigated in a subset of participants (*n*=40 men and *n*=40 women) by discovery proteomics after fast-protein liquid chromatography isolation and lipid removal agent enrichment of large (L-HDL) and small HDL (S-HDL)-containing fractions.

Results: Total CEC was significantly increased in non-obese women relative to men, coincident with an increase in L-HDL-P, and reduction in S-HDL-P, relative to men. By contrast, HDL from women with T2DM exhibited significantly reduced capacity to support total CEC and ABCA1-dependent efflux, and reduced concentration of S-HDL-P, compared to men with T2DM. There were no differences in HDL-CEC between women and men with T1DM. L-HDL-P from women (all groups combined) were enriched with pregnancy zone protein (PZP), ApoA-I, ApoA-II, SAA2-4 and depleted of phosphatidylinositol-glycan specific phospholipase D (GPLD1) and beta-2-glycoprotein 1 (APOH) relative to men. By contrast, ApoA-I, ApoC-I, ApoC-II, ApoC-III, ApoD, ApoM, vitamin K-dependent protein S (PROS1) and bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4 (NDST4) were depleted on S-HDL-P in women relative to men. The proteins governing HDL-CEC were significantly different between the sexes. Apolipoproteins, ApoA-I, ApoA-II, ApoC-I, ApoC-III, ApoC-IV, ApoD, ApoE, ApoF and ApoM, were more dominant drivers of CEC in women compared to men, while complement proteins, C3/C5 convertase, C4b-binding protein alpha chain, complement component alpha, beta and gamma chain and complement component 9, were positive drivers of CEC in men compared to women. Of interest, association of Igs on HDL were negatively associated with CEC in men but positively associated in women.

Conclusion: Our findings highlight that women with both T1DM and T2DM are disadvantaged in terms of HDL-CEC relative to male counterparts. We also report sex-specific differences in the proteins governing HDL-CEC in women versus men that may have important implications for their respective cardiovascular risk profile. Indeed, our findings indicate the use of separate biomarkers to predict HDL function in men and women.

Abbreviations

ABCA1	ATP-Binding Cassette Subfamily A Member 1
ABCG1	ATP-binding Cassette subfamily G, member 1
ACE-I	Angiotensin-Converting Enzyme Inhibitors
ACPS	Serum Amyloid Component P
ANOVA	Analysis of Variance
Apo	Apolipoprotein
ApoA-I	Apolipoprotein A-I
APOH	Beta-2-Glycoprotein 1
ARB	Angiotensin Receptor Blockers
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
CEC	Cholesterol Efflux Capacity
CETP	Cholesterol Ester Transfer Protein
CHD	Coronary Heart Disease
CI	Confidence Interval
CVD	Cardiovascular Disease
DPP4	Dipeptidyl Peptidase-4
FDR	False Discovery Rate
FPLC	Fast Protein Liquid Chromatography
GLP-1	Glucagon-like Peptide-1
GPLD1	Phosphatidylinositol-Glycan-Specific Phospholipase D
HbA1c	Haemoglobin A1c
HDL	High-Density Lipoprotein
HDL-C	High-Density Lipoprotein-Cholesterol
HDL-P	High-Density Lipoprotein-Particle
HR	Hazard Ratio
Ig	Immunoglobulin
ITIH	Inter-Alpha-Trypsin Inhibitor Heavy Chain H
LCAT	Lecithin-Cholesterol Acyltransferase/ Phosphatidylcholine-sterol Acyltransferase
LDL	Low-Density Lipoprotein
LDL-C	Low-Density Lipoprotein-Cholesterol
LFQ	Label-Free Quantification
LRA	Lipid Removal Agent
MS	Mass Spectrometry
NDST4	Bifunctional Heparan Sulfate N-deacetylase/N-sulfotransferase 4

NMR	Nuclear Magnetic Resonance
OB	Obesity/Obese
PON1	Paraoxonase 1/ Serum Paraoxonase/Arylesterase 1
PLTP	Phospholipid Transfer Protein
PROS1	Vitamin K-Dependent Protein S
PZP	Pregnancy Zone Protein
RCT	Reverse Cholesterol Transport
RR	Relative Risk
SAA1	Serum Amyloid A 1
SD	Standard Deviation
SHBG	Sex Hormone Binding Globulin
SR-B1	Scavenger Receptor Class B Type 1
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides

5.1 Introduction

Women without diabetes are relatively protected from cardiovascular disease (CVD) compared to men without diabetes, with lower mortality rates from coronary heart disease (CHD) evident [1]. A global assessment of sex differences in CHD mortality found men aged 30-60 years had approximately a four times higher mortality rate compared to women of similar ages [1]. Additionally, women tend to develop CVD 7-10 years later than men [2]. However, the cardioprotective benefits associated with being a women are negated in women with diabetes through as yet unknown mechanisms. A US study involving 116,177 women, aged 30-55, found that women with type 2 diabetes mellitus (T2DM) have a 3 fold excess risk of fatal coronary heart disease (95% CI, 1.9, 4.8) compared to women without T2DM [3]. Likewise, women with T2DM have a higher adjusted hazard ratio (HR) of fatal CAD (HR 14.74; 95% CI, 6.16, 35.27) compared to men with T2DM (HR 3.77; 95% CI, 2.52, 5.65) [4]. A meta-analysis, involving 102 prospective studies, revealed that the risk of CHD is higher in women with diabetes, HR 2.59 (95% CI, 2.29, 2.93), compared to men with diabetes, HR 1.89 (95% CI, 1.73, 2.06), $P<0.0001$ and that the risk was also higher among those of a younger age (40-59 years) [5]. Additionally, another study investigating both type 1 diabetes mellitus (T1DM) and T2DM as risk factors for heart failure also found that women with diabetes were more severely impacted by their disease compared to men. The multiple adjusted relative risk (RR) for heart failure associated with T2DM was 1.95 (95% CI, 1.70, 2.22) in women and 1.74 (95% CI, 1.55, 1.95) in men. Similarly, the corresponding RR associated with T1DM were 5.15 (95% CI 3.43, 7.74) for women and 3.47 (95% CI 2.57, 4.69) for men [6]. As a result of the increased risk of CVD in women with diabetes, this has translated into more deaths associated with diabetes in women (2.3 million) than in men (1.9 million) worldwide [7].

Traditionally, there has been a male predominance during preclinical (cellular and animal models) and clinical research efforts, hindering the ability to elucidate the potential pathogenic mechanisms of diabetes and CVD in women versus men. Addressing sex differences has emerged as a priority topic in several medical fields including metabolic disease [8].

In the clinical setting, standard lipid analysis includes measurement of fasting serum or plasma total cholesterol, triglycerides (TG), high-density lipoprotein (HDL)-cholesterol (HDL-C), and low-density lipoprotein (LDL)-cholesterol (LDL-C) [9]. However, these relatively simple lipid measurements potentially mask more subtle lipoprotein abnormalities, including disorders of lipoprotein function, that might contribute differentially to the residual CVD risk in women and men with diabetes. Data from the Pittsburgh Epidemiology of Diabetes Complications Study report that the usual inverse association between HDL-C and CHD risk, although retained in men (HR 0.97, 95% CI 0.94, 0.99), is altered in women with T1DM (U shaped trend), who

demonstrate little increased protection with concentrations above the range of 50 to 60 mg/dL, suggesting that potentially in women with T1DM, HDL is dysfunctional [10]. The size of HDL particles (HDL-P) is also important in accessing their anti-atherogenic properties. Multiple studies have found that higher small HDL-P (S-HDL-P) are most strongly associated with lower CVD risk [11-13]. However, other studies show the opposite and, by extension suggest that large HDL-P (L-HDL-P) are the more protective subclass [14, 15]. These studies vary in design, adjustment for confounders, and methods for HDL subpopulation isolation and quantification, hence, there are conflicting data concerning HDL subpopulations and subsequent prediction of CVD. Additionally, most of these studies only recruited men, or combined women and men and did not directly compare men and women. Men tend to have a less favourable lipoprotein-lipid profile potentially accounting for their higher CVD risk [16]. Overall, these contrasting observations, highlight that a more comprehensive understanding of HDL biology is needed, in women and men with and without T1DM or T2DM.

HDL-P are complex carrying over 150 lipids [17], 251 proteins [18], and 304 microRNAs [19] – this heterogeneity translates into several distinct HDL subspecies with potentially different functional properties. The complexity of HDL-P composition, and the multitude of HDL subfractions, is a major challenge for unravelling structure-function relationships. HDL-P exert numerous atheroprotective functions including antioxidant, anti-inflammatory and cholesterol efflux promoting functions [20]. The central role of HDL in reverse cholesterol transport (RCT), is widely considered the key mechanism responsible for HDL-mediated cardio-protection. In the RCT pathway, HDL accepts cholesterol from peripheral cells including lipid-laden foam cells within atherosclerotic lesions for delivery to the liver, and subsequent elimination in the bile and faeces [21, 22]. HDL-cholesterol efflux capacity (HDL-CEC) is driven by the interaction with cellular cholesterol transporters including ATP binding cassette subfamily A, member 1 (ABCA1), ATP-binding cassette subfamily G, member 1 (ABCG1) and scavenger receptor class B member 1 (SR-B1). In macrophages, small dense lipid-poor HDL-P potently promote efflux via the ABCA1 transporter, while larger HDL-P interact with ABCG1 and SR-B1 transporters [23], and thus different sized particles interact with specific efflux pathways. Measurement of HDL-CEC, as opposed to static HDL-C levels, is now acknowledged as a stronger predictor of CVD risk [24-26], highlighting that HDL quality seems to be more important than its quantity. There are no studies to our knowledge that have comprehensively compared HDL structure, function, and composition in men versus women to gain a greater understanding of sex-specific differences underpinning CVD pathogenesis between the sexes.

Given the conflicting data surrounding the association of L-HDL-P and S-HDL-P with CVD, and that women and men have different lipoprotein profiles, it is pertinent that we get a better

understanding of the biology underpinning these different sized particles in both sexes. It is furthermore imperative that we gain a better understanding of sex-specific differences in HDL biology that may contribute to the global 'female disadvantage' in diabetes.

Within this study we sought to understand the impact of sex on HDL functionality and HDL composition. We investigated sex-specific differences in HDL-CEC functionality and how the size and protein composition are differentially affected in women and men with and without T1DM or T2DM.

Study hypotheses:

1. HDL protein composition will be significantly different between men and women contributing to differing CVD pathogenesis between the sexes.
2. Proteins governing HDL-CEC will differ between the sexes.
3. Women with diabetes will exhibit specific greater dysfunction in their HDL-P relative to men contributing to exacerbated CVD risk.

Objective:

To elucidate differences in HDL biology between women and men with and without T1DM or T2DM.

Aims:

The aims of this study were:

1. To assess whether there are sex-specific differences in total CEC, ABCA1-dependent CEC and ABCA1-independent CEC in people with and without T1DM or T2DM.
2. To assess whether there are sex-specific differences in HDL-P size and subsequent relationship to HDL-CEC.
3. To investigate the HDL proteome of the L-HDL-P and S-HDL-P in women and men with and without T1DM or T2DM.
4. To investigate sex-specific differences in HDL proteomic composition and subsequent relationship to HDL-CEC.

5.2 Methods

5.2.1 Study design and participants

This was a case-control study designed to compare HDL size, HDL proteomic composition and HDL-CEC between women and men with and without T1DM or T2DM. A sub-cohort of participants from the previous chapter were chosen for this study's analysis.

Women and men with T1DM (*n*=20 per group), and women and men with T2DM (*n*=20 per group) were age matched, and their respective controls were age and BMI matched. Two control groups (*n*=20 per control group per sex) were selected as there is a disconnect in BMI between T1DM and T2DM, with people with T2DM generally having a higher BMI. The study design is presented in **Figure 5.1**.

Participants with T1DM or T2DM were contacted either by phone or at the time of their scheduled clinic visit. Inclusion criteria for T1DM were as follows: age 20–65 years; BMI ≥18.5 kg/m^2; duration of diabetes of at least 1 year and HbA1c >6.5%. Inclusion criteria for T2DM were as follows: age 20–65 years; BMI ≥18.5 kg/m^2; duration of diabetes of at least 1 year and HbA1c >6.5%. Exclusion criteria were as follows: pregnant or lactating; and recent illness or any chronic illness likely to influence results. Each participant with diabetes was pair-matched with a control volunteer, recruited from the general population. Inclusion criteria were as follows: non-diabetic, age 20–65 years; BMI ≥18.5 kg/m^2; and HbA1c ≤6%. Exclusion criteria were as for participants with diabetes. The Diabetes Day Centre in Tallaght University Hospital (TUH), Dublin, recruited all participants with diabetes. Control samples were obtained from TUH and the obesity clinic at St. Vincent's University Hospital (SVUH), Dublin.

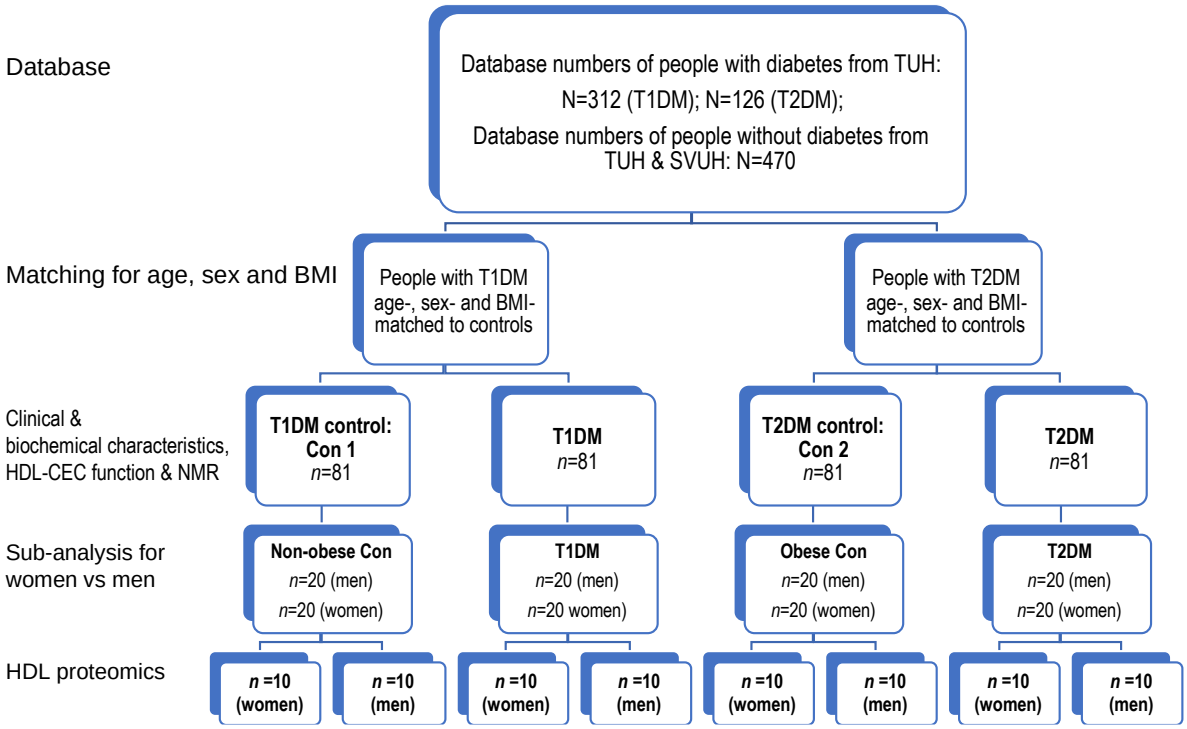

Figure 5.1 *Study design*

5.2.2 Ethical Approval

All participants gave their written informed consent, which was approved by the Research Ethics Committee of TUH (Dublin, Ireland) & SVUH (Dublin, Ireland).

5.2.3 Study protocol and anthropometric data

All participants were studied after a 12 h fast, having avoided excessive exercise and alcohol for the previous 24 h. Height was measured with a Harpenden stadiometer. Weight was measured in a hospital gown. Completed in TUH and SVUH.

5.2.4 Laboratory methods

Plasma levels of total cholesterol, TG and HDL-C were measured by an enzymatic calorimetric method on the Roche P Module (Roche, Mannheim, Germany). LDL-C was calculated using the Friedewald equation. Additional samples were centrifuged at 3000 rpm for 15 min at 4°C, and serum were stored at −80°C. Completed in TUH and SVUH.

5.2.5 Cholesterol efflux capacity (CEC) [27]

J774.2 murine macrophages, derived from BALB/c mice (European Collection of Authenticated Cell Cultures), were seeded (7×10^5 cells/ml) onto 96-well plates for 24 h, and cultured at 37°C. Cells were subsequently labelled with ^{3}H-cholesterol (1 μCi/ml) for 24 h

before being equilibrated for 18 h in DMEM containing 0.2% BSA with or without cAMP (0.3 mmol/l). J774 macrophages do not express ABCA1 basally, and stimulation with cAMP specifically drives ABCA1 protein expression. ApoB-containing lipoproteins were removed from serum by polyethylene glycol (PEG) precipitation, leaving a HDL-enriched supernatant fraction. *Ex vivo* efflux from labelled macrophages to 2.8% HDL-enriched supernatant fraction in minimal essential media (MEM) was measured over 4 h. Control cells were incubated with MEM alone to control for non-specific efflux. ^{3}H-cholesterol levels in the media and remaining cells were calculated by liquid scintillation counting for determination of percentage efflux. Total CEC was determined from cells treated +cAMP. The difference in efflux to HDL from cells stimulated in the presence and absence of cAMP was taken to represent ABCA1-dependent CEC, while ABCA1-independent CEC was calculated from cAMP naive cells.

5.2.6 Nuclear magnetic resonance (NMR) [27]

Lipoprotein particles concentration and sizes were measured in serum samples using 400 MHZ NMR spectroscopy (LipoScience, Raleigh, NC, USA). Each lipoprotein subclass emits a distinctive lipid methyl group NMR signal and the amplitudes of the measured signals are directly proportional to the number of subclass particles, which in turn provides the estimated mol/l concentration for the three HDL-P subclasses (large 8.9 – 13 nm, medium 8.3 – 8.8 nm, small 7.3 – 8.2 nm) [28]. Weighted-average HDL-P sizes in nanometres were calculated from the subclass levels, and the diameters were assigned to each subclass.

5.2.7 HDL isolation via fast-protein liquid chromatography (FPLC)

Lipoprotein subpopulations (VLDL, LDL, HDL) were separated within human serum, from a sub cohort of *n*=10 per group per sex, via FPLC. 100 µl of sample, at a serum to PBS containing 1 mM EDTA ratio of 1:1, was injected into the injection valve of the ÄKTA FPLC system (GE Healthcare). The sample processed through two Superose 6 Increase 10/300 GL columns (GE Healthcare, Sweden) placed sequentially, and was eluted into microcentrifuge tubes as 44 separate fractions using a Frac 920 collector (GE Healthcare). Cholesterol content of each freshly eluted fraction was measured by commercial assay (LabAssay™ Cholesterol; Fujifilm) as per manufacturer's instructions. Fractions were stored at -80°C.

5.2.8 Proteomics

Sample preparation for proteomics is described in detail in **Chapter 2, Section 2.7.**

5.2.8.1 Protein isolation

HDL containing fractions 36-38 (large HDL particles, L-HDL-P), and fractions 40-42 (small HDL particles, S-HDL-P) were separately pooled and purified using 30 µl lipid removal agent (LRA)

(100mg/ml in 50mM ammonium bicarbonate, Sigma-Aldrich, Ireland) [29] to further enrich for phospholipid-containing HDL-P in the fractions. The LRA pellet was resuspended in 25 µl of 50 mM ammonium bicarbonate containing 2 M Urea.

5.2.8.2 In-solution digestion

Protein samples were in-solution digested with trypsin, (1 vial of trypsin singles at 1 µg size, proteomic grade T7575, Sigma Aldrich, per two samples) at a protein to trypsin ratio of 50:1 (v/v), overnight at 37°C. Peptides were acidified with formic acid (FA) to stop trypsin digestion. Tryptic digests were purified using C_{18}ZipTip (Millipore) following the manufactures instructions, and resuspended in 3% acetonitrile (ACN) with 0.1% FA, prior to mass spectrometry (MS) analysis.

5.2.8.3 Mass Spectrometry

Peptides were analysed on a quadripole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer fitted with a reversed-phase nano-LC UltiMate 3000 high performance liquid chromatography (HPLC) system (Thermo Scientific).

5.2.8.4 Protein identification

Raw data were processed using the MaxQuant version 1.6.10.43, incorporating the Andromeda search engine. To identify peptides and proteins, MS/MS spectra were matched to the Uniprot *homo sapiens* database (2020_06) containing 76,074 entries. In order to generate label free quantitative (LFQ) ion intensities for protein profiles, signals of corresponding peptides in different nano-HPLC MS/MS runs were matched by MaxQuant applying a mass accuracy of at least 20 ppm and a maximum time window of 1 min [30].

5.2.8.5 Proteomic data analysis

Perseus statistical software version 1.6.5.0 was used to analyse the LFQ intensities. Data were log transformed, and t-test comparisons of samples/groups conducted. For visualization using heat maps, missing values were imputed with values from a normal distribution, and the z-score was used to normalise the dataset.

5.2.9 Statistical Analysis

Results in tables are presented as mean ± SD unless otherwise stated. Data in graphs are presented as mean ± SD. Data were tested for normality using the Shapiro–Wilk test, as well as inspected visually using histograms. Statistical significance for categorical variables was calculated using the χ^2 test. For continuous variables of normally distributed data, the unpaired Student's t test was used to assess statistical significance of men versus women, while for non-normally distributed data the Mann-Whitney U t-test was applied. For multiple comparisons on normal data, between sub-groups of women and men separately, a paired one-way analysis of variance (ANOVA) test was performed with a Bonferroni post-test. The

Kruskal-Wallis test with a Dunn's multiple comparison post-test was performed on non-normal data. Correlations were evaluated using the Pearson correlation (normal data) or Spearman test (non-normal data) as appropriate.

IBM SPSS Statistics (Version 24.0. Armonk, NY) and GraphPad Prism (Version 9.3.0 San Diego, CA) software for Mac were used for data analysis. Statistical significance is presented

$^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$ for non-obese control vs. T1DM;

$^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$, $^{\$\$\$\$}P<0.0001$ for non-obese control vs. obese control;

$^{\bowtie}P<0.05$, $^{\bowtie\bowtie}P<0.01$, $^{\bowtie\bowtie\bowtie}P<0.001$, $^{\bowtie\bowtie\bowtie\bowtie}P<0.0001$ for non-obese control vs. T2DM;

$^{\S}P<0.05$, $^{\S\S}P<0.01$, $^{\S\S\S}P<0.001$, $^{\S\S\S\S}P<0.0001$ for obese control vs. T2DM; and

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ for T1DM vs. T2DM.

$P>0.05$ was considered non-significant.

5.3 Results

5.3.1 Baseline characteristics of women and men with or without T1DM or T2DM

Clinical and biochemical characteristics of T1DM, T2DM and non-diabetic participants (*n*=20 per group) for women and men are shown in **Table 5.1** and direct comparisons are shown in **Table 5.2**. The medication use in the four groups is shown in **Table *5.9*** in **Appendix 5A – Medication Tables.**

All four groups were age matched. HbA1c was significantly higher in people with T1DM and T2DM compared to controls in both women and men. HDL-C levels were significantly reduced in women with T2DM relative to the non-obese controls, with a non-significant increase in HDL-C evident in T1DM compared the non-obese controls. There was no difference in HDL-C levels in men with T2DM relative to their non-obese controls. A similar trend towards increased HDL-C levels was evident in men with T1DM compared to their non-obese controls as was observed in women. It is noteworthy that levels of HDL-C were higher in women across all groups relative to men, except for women with T2DM who showed parity in HDL-C levels as men with T2DM (**Table 5.2**). It is also noteworthy that men with obesity, and T2DM exhibited significantly higher systemic TG levels relative to women.

Table 5.1 *Baseline characteristics of women and men with T1DM and T2DM and their respective controls.*

Characteristic	Non-OB (*n*=20)	T1DM (*n*=20)	OB (*n*=20)	T2DM (*n*=20)	Non-OB (*n*=20)	T1DM (*n*=20)	OB (*n*=20)	T2DM (*n*=20)
	Women				*Men*			
Age (years)	41.3 ± 6.6	41.2 ± 6.5	39.8 ± 5.8	40.2 ± 5.4	41.8 ± 7.3	42.7 ± 7.4	42.8 ± 7.9	43.4 ± 6.9
Body mass index (kg/m^2)	25 ± 2.9	24.5 ± 2.5	35.8 ± 4.2$^{\$\$\$\$}$	35.7 ± 4.4$^{⋈⋈⋈⋈;****}$	25.5 ± 1.9	25.4 ± 2.5	35.5 ±5.8$^{\$\$\$\$}$	36.1 ± 4.9$^{⋈⋈⋈⋈;****}$
Waist to hip ratio	0.84 ± 0.06	0.82 ± 0.07	0.88 ± 0.07	0.92 ± 0.05$^{⋈⋈,**}$	0.91 ± 0.07	0.90 ± 0.06	1.0 ± 0.04$^{\$\$}$	1.0 ±0.04$^{⋈⋈⋈⋈,****}$
Smokers (%)	15	30	20	20	10	20	15	15
Systolic blood pressure (mm Hg)	126 ± 14	127 ± 17	127 ± 16	122 ± 12	132 ±11	135 ± 12	132 ±12	132 ± 12
Diastolic blood pressure (mm Hg)	81 ± 11	76 ± 5	85 ± 8	79 ± 13	80 ± 7	83 ± 8	82 ± 11	80 ± 7
Haemoglobin A1c (%)	5.3 ± 0.2	7.9 ± 1$^{####}$	5.4 ± 0.3	7.3 ± 1.8$^{⋈⋈;**}$	5.4 ± 0.2	8.2 ± 1$^{####}$	5.4 ± 0.3	7.8 ± 1.6$^{⋈⋈;***}$
Total cholesterol (mmol/l)	4.5 ± 1.4	4.5 ± 0.6	5.3 ± 0.9	4.1 ± 1§§	5.1 ± 0.6	4.3 ± 1$^{\#}$	5.5 ± 1.2	5.2 ± 2.8
Triglycerides (mmol/l)	1 (0.6-1.4)	0.8 (0.7-1.2)	1.1 (1-1.7)	1.5 (0.9-2)*	0.9 (0.7-1.2)	0.9 (0.7-1.2)	1.6 (1.3-3.1)$^{\$}$	2.5 (1.5-3.4) $^{⋈⋈;***}$
LDL cholesterol (mmol/l)	2.5 ± 1	2 ± 0.9	3.4 ± 0.8	2.5 ± 0.7§§	3.2 ± 0.5	2.3 ± 0.8$^{\#\#\#}$	3.4 ± 1.1	2.4 ± 1.1
HDL cholesterol (mmol/l)	1.7 ± 0.5	1.9 ± 0.5	1.4 ± 0.4	1.1 ± 0.2$^{⋈;****}$	1.4 ± 0.3	1.6 ± 0.4	1.1 ± 0.2$^{\$\$}$	1.1 ± 0.5*

Data are displayed as mean ± SD, median (IQR) or %
Statistical significance is presented as
$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, $^{\#\#\#\#}P$<0.0001 for Con1 vs. T1DM;
$^{\$}P$<0.05, $^{\$\$}P$<0.01, $^{\$\$\$}P$<0.001, $^{\$\$\$\$}P$<0.0001 for Con1 vs. Con2;
$^{⋈}P$<0.05, $^{⋈⋈}P$<0.01, $^{⋈⋈⋈}P$<0.001, $^{⋈⋈⋈⋈}P$<0.0001 for Con1 vs. T2DM;
$^{\S}P$<0.05, $^{\S\S}P$<0.01, $^{\S\S\S}P$<0.001, $^{\S\S\S\S}P$<0.0001 for Con2 vs. T2DM; and
*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 for T1DM vs. T2DM.

Table 5.2 *Baseline characteristics of women and men compared across the four groups.*

Characteristic	Non-OB (*n*=20)	Non-OB (*n*=20)	T1DM (*n*=20)	T1DM (*n*=20)	OB (*n*=20)	OB (*n*=20)	T2DM (*n*=20)	T2DM (*n*=20)
	Women	Men	Women	Men	Women	Men	Women	Men
Age (years)	41.3 ± 6.6	41.8 ± 7.3	41.2 ± 6.5	42.7 ± 7.4	39.8 ± 5.8	42.8 ± 7.9	40.2 ± 5.4	43.4 ± 6.9
Body mass index (kg/m^2)	25 ± 2.9	25.5 ± 1.9	24.5 ± 2.5	25.4 ± 2.5	35.8 ± 4.2	35.5 ±5.8	35.7 ± 4.4	36.1 ± 4.9
Waist to hip ratio	0.84 ± 0.06	0.91 ± 0.07**	0.82 ± 0.07	0.90 ±0.06**	0.88 ± 0.07	1.0 ± 0.04****	0.92 ± 0.05	1.0 ± 0.04****
Smokers (%)	15	10	30	20	20	15	20	15
Systolic blood pressure (mm Hg)	126 ± 14	132 ±11	127 ± 17	135 ± 12	127 ± 16	132 ±12	122 ± 12	132 ± 12*
Diastolic blood pressure (mm Hg)	81 ± 11	80 ± 7	76 ± 5	83 ± 8**	85 ± 8	82 ± 11	79 ± 13	80 ± 7
Haemoglobin A1c (%)	5.3 ± 0.2	5.4 ± 0.2	7.9 ± 1	8.2 ± 1	5.4 ± 0.3	5.4 ± 0.3	7.3 ± 1.8	7.8 ± 1.6
Total cholesterol (mmol/l)	4.5 ± 1.4	5.1 ± 0.6	4.5 ± 0.6	4.3 ± 1	5.3 ± 0.9	5.5 ± 1.2	4.1 ± 1	5.2 ± 2.8
Triglycerides (mmol/l)	1 (0.6-1.4)	0.9 (0.7-1.2)	0.8 (0.7-1.2)	0.9 (0.7-1.2)	1.1 (1-1.7)	1.6 (1.3-3.1)*	1.5 (0.9-2)	2.5 (1.5-3.4) *
LDL cholesterol (mmol/l)	2.5 ± 1	3.2 ± 0.5*	2 ± 0.9	2.3 ± 0.8	3.4 ± 0.8	3.4 ± 1.1	2.5 ± 0.7	2.4 ± 1.1
HDL cholesterol (mmol/l)	1.7 ± 0.5	1.4 ± 0.3	1.9 ± 0.5	1.6 ± 0.4*	1.4 ± 0.4	1.1 ± 0.2*	1.1 ± 0.2	1.1 ± 0.5

Data are displayed as mean ± SD, median (IQR) or %
Statistical significance is presented as
*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 for women vs. men.
Non-OB = Non-obesity, T1DM = type 1 diabetes mellitus; OB = obesity; T2DM = type 2 diabetes mellitus.

5.3.2 Total CEC is impaired in women with T2DM, and enhanced in men with T1DM

Non-obese women exhibited significantly increased total CEC, relative to BMI-matched non-obese men (**Figure 5.2 B**).

By contrast, women with T2DM exhibited significantly reduced total CEC relative to men, which was largely due reductions in ABCA1-dependent efflux (**Figure 5.2 B, D**).

Women with T2DM similarly exhibited significantly reduced total CEC compared to their BMI-matched obese controls, but this wasn't apparent in men with T2DM, who showed a trend towards increased efflux capacity compared to their obese controls.

Men with T1DM exhibited significantly increased total CEC compared to their non-obese controls, which was largely attributable to an increase in ABCA1-independent efflux. This benefit was not observed in women with T1DM, highlighting a disadvantage in women in both T1DM and T2DM (**Figure 5.2 A, C & E**). No significant difference in HDL-CEC was evident between men and women with T1DM (**Figure 5.2 B, D, F**).

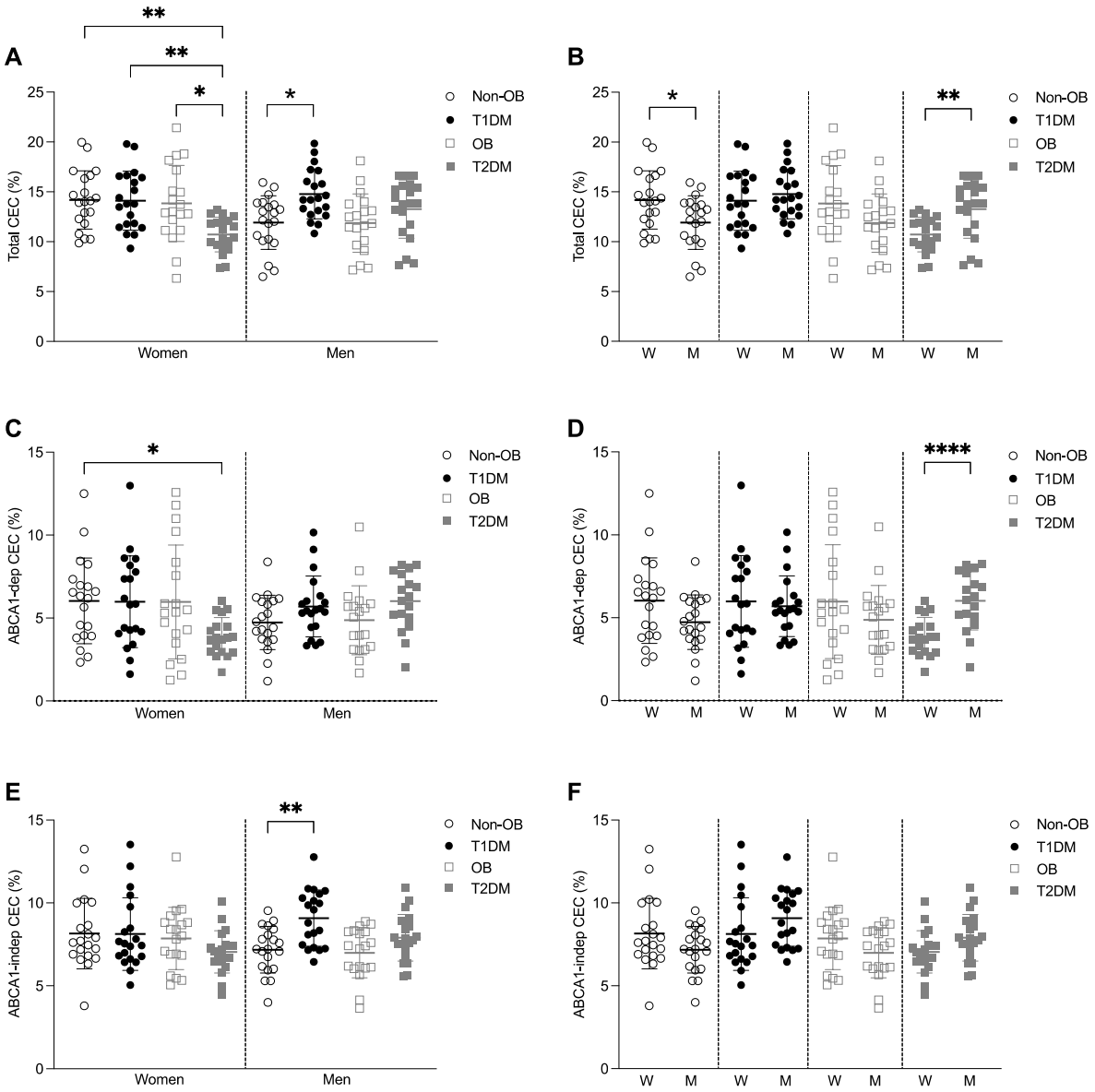

Figure 5.2 *HDL functionality in women and men with and without T1DM or T2DM and their respective controls.*

HDL-CEC measurements in women and men with and without T1DM or T2DM and their respective controls (*n*=20 per group). J774 macrophages were loaded with ^{3}H-Cholesterol (1µCi/ml) for 24h. Cells were then treated ± cAMP (0.3mM) for 16h and efflux to 2.8% PEG supernatant was monitored over 4h. Dot plot shows results for **(A&B)** total CEC, derived from +cAMP treated cells, **(C&D)** ABCA1-dependent CEC, calculated as the difference in efflux to PEG-supernatant between cells treated with/ without cAMP**,** and **(E&F)** ABCA1-independent CEC was calculated from –cAMP treated cells. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Non-OB = non-obesity; OB = Obesity; W = women; M = men.

5.3.3 HDL-P size is differentially modulated in men and women with and without T1DM or T2DM

Women with T1DM, and corresponding non-obese controls, exhibited significantly higher concentrations of L- HDL-P relative to men (**Figure 5.3 B**). A significant reduction in L-HDL-P concentration is evident in women with obesity relative to women without obesity. Similarly, a significant reduction in L-HDL-P concentration is evident in women with T2DM relative to women with T1DM. By contrast, there is no difference in L-HDL-P concentration between men with obesity versus non-obese controls. Consistent with findings in women, a significant reduction in the concentration of L-HDL-P concentration is evident in men with T2DM relative to men with T1DM (**Figure 5.3 A**).

On the contrary, the concentration of S-HDL-P is significantly reduced in women with T1DM relative to non-obese controls and also relative to women with T2DM. Similar trends were evident in S-HDL-P concentration in men with T1DM or T2DM relative to their respective controls but failed to reach significance (**Figure 5.3 C**). A significant increase in the concentration of S-HDL-P was evident in men relative to women across all groups, with the exception of the obesity group, indicative of sex specific differences in HDL-remodelling (**Figure 5.3 D**). No difference in medium sized particles was evident between men and women or across categories of metabolic disease (data not shown).

It is noteworthy that despite reduced levels of HDL-C in men versus women, the concentration of HDL-P was significantly increased in men with T1DM, and men with obesity relative to women in those groups (**Figure 5.3 F**). Finally, consistent with a higher-concentration of larger HDL-P, the average diameter of HDL-P was significantly higher in women relative to men without obesity, and with T1DM (**Figure 5.3 H**); these differences in HDL-P size are negated in women with obesity or T2DM, which was consistent with a reduction in the concentration of L-HDL-P in these groups.

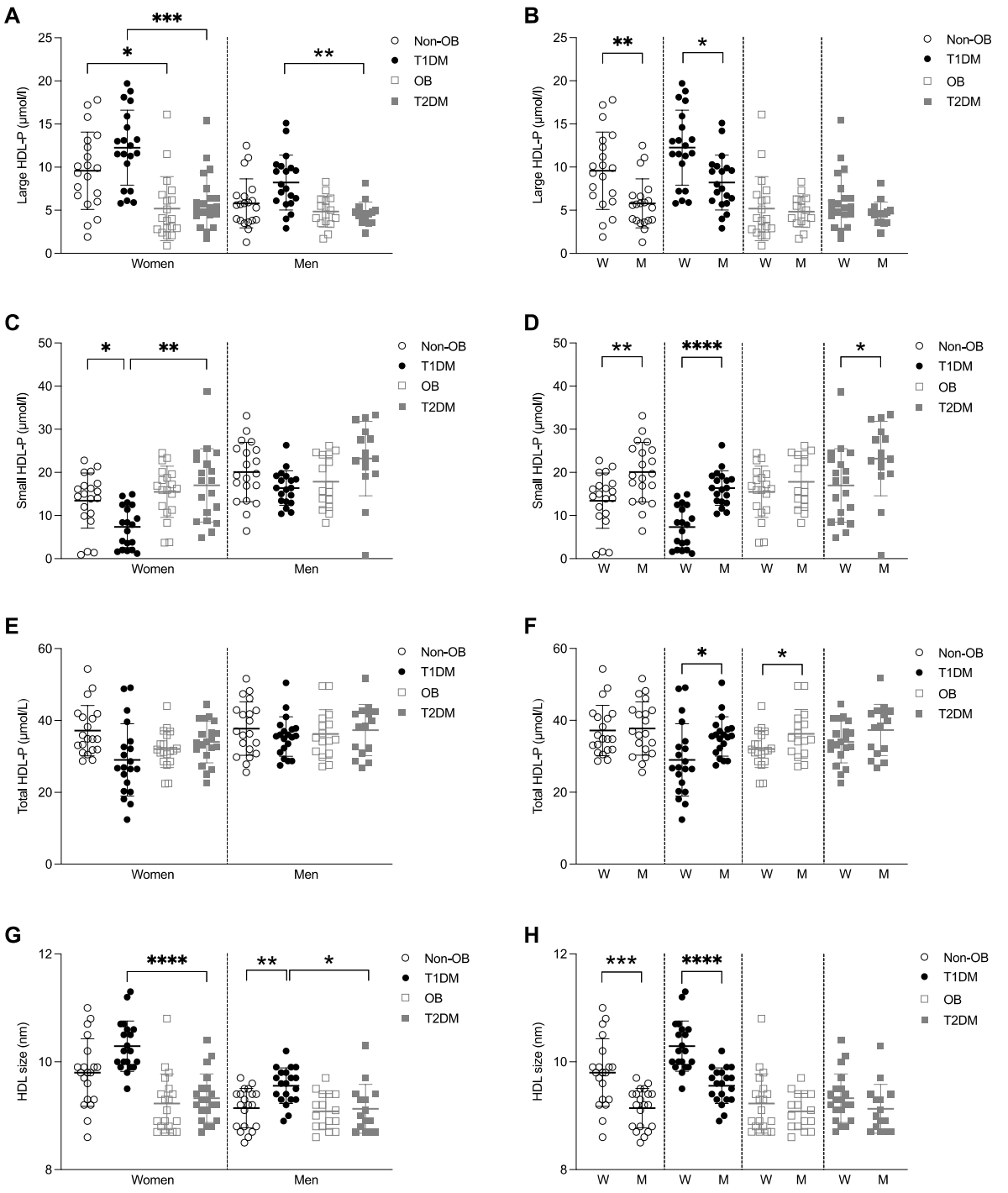

Figure 5.3 *HDL concentration and HDL-size.*

HDL composition analysis in women and men with and without T1DM or T2DM and their respective controls. 150µL plasma was sent for NMR analysis to LabCorp (LipoScience Inc, NC, USA) (*n*=10 per group). Dot plot shows results for **(A&B)** Large HDL-P, **(C&D)** Small HDL-P, **(E&F)** Total HDL-P, and **(G&H)** HDL-P diameter.

Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.Non-OB = non-obesity; OB = Obesity; W = women; M = men.

5.3.4 Correlations of HDL-CEC function with HDL concentration, size and cholesterol content in women and men

We subsequently investigated the relationship between HDL-CEC, and HDL-P concentration, size and cholesterol content in women and men with T1DM or T2DM and their respective controls.

Across the total population combined, we identified significant positive correlations between total CEC and L-HDL-P concentration, total HDL-P concentration, and HDL size in both women and men. A significant positive correlation between total CEC and HDL-C levels was also apparent, which was stronger in women (r=0.554) compared to men (r=0.259). Similar trends in correlations were also evident for ABCA1-independent CEC. A stronger correlation between ABCA1-independent CEC and total HDL-P concentration was evident in women (r=0.531) compared to men (r=0.302), while a stronger correlation between ABCA1-independent CEC and HDL size was evident in men (0.462) compared to women (r=0.277). There was also a significant correlation between ABCA1-independent CEC and M-HDL-P (r=0.469) in men which was not evident in women (r=0.189 ns). The only significant associations with ABCA1-dependent CEC were to HDL size and HDL-C levels in women only. The significant associations between HDL-CEC and HDL-P size within the sub-groups are expanded on in **Table 5.3**.

Table 5.3 *Correlations of HDL-CEC parameters with HDL concentration, size and cholesterol content.*

Characteristics	All (*n*=80)		Non-OB (*n*=20)		T1DM (*n*=20)		OB (*n*=20)		T2DM (*n*=20)	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
	r	r	r	r	r	r	r	r	r	r
Total CEC										
Large HDL-P concentration (μmol/l)	0.460****	0.420***	0.141	0.190	0.597**	0.414	0.618**	0.638**	0.309	−0.039
Medium HDL-P concentration (μmol/l)	0.135	0.195	0.365	−0.047	0.368	0.210	−0.020	0.652**	−0.084	0.042
Small HDL-P concentration (μmol/l)	−0.119	−0.064	0.062	0.067	−0.176	−0.104	−0.155	−0.322	0.331	0.305
Total HDL-P concentration (μmol/l)	0.281*	0.277*	0.445*	0.118	0.398	0.345	0.231	0.670**	0.593**	0.390
HDL size (nm)	0.390***	0.352**	0.123	0.292	0.425	0.206	0.525*	0.476	0.176	−0.145
HDL cholesterol (mmol/l)	0.554****	0.259*	0.231	0.152	0.456*	0.445	0.774***	0.019	0.177	0.074
ABCA1-dependent CEC										
Large HDL-P concentration (μmol/l)	0.218	0.111	−0.214	0.008	0.314	−0.047	0.421	0.328	−0.110	−0.297
Medium HDL-P concentration (μmol/l)	−0.170	0.134	0.082	−0.230	−0.299	0.036	−0.240	0.546*	−0.346	0.167
Small HDL-P concentration (μmol/l)	−0.033	−0.001	0.160	−0.010	−0.409	0.126	0.122	−0.100	0.454*	0.122
Total HDL-P concentration (μmol/l)	−0.043	0.159	0.077	−0.101	−0.252	0.128	0.114	0.660**	0.233	0.202
HDL size (nm)	0.260*	0.127	−0.064	0.169	0.439	−0.284	0.399	0.336	−0.178	−0.085
HDL cholesterol (mmol/l)	0.315**	−0.004	0.224	0.004	−0.065	0.031	0.614**	−0.083	−0.368	−0.058
ABCA1-independent CEC										
Large HDL-P concentration (μmol/l)	0.455****	0.594****	0.454*	0.353	0.410	0.660**	0.472*	0.749***	0.523*	0.341
Medium HDL-P concentration (μmol/l)	0.469****	0.189	0.403	0.177	0.876****	0.269	0.397	0.555*	0.266	−0.154
Small HDL-P concentration (μmol/l)	−0.152	−0.106	−0.110	0.142	0.278	−0.288	−0.532*	−0.445	−0.050	0.417
Total HDL-P concentration (μmol/l)	0.531****	0.302*	0.519*	0.345	0.856****	0.369	0.255	0.472	0.550*	0.468
HDL size (nm)	0.277*	0.462****	0.248	0.360	0.019	0.609**	0.325	0.468	0.438	−0.161
HDL cholesterol (mmol/l)	0.475****	0.393**	0.005	0.277	0.698***	0.620**	0.438	0.170	0.626**	0.066

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001,

Correlation coefficient (r)

+1 Perfect
+ 0.800 - 0.999 V. strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 V. Weak
Non-sig
−0.000 - −0.199 V. Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 V. strong
−1 Perfect

5.3.5 Baseline characteristics of sub-cohort for proteomic analysis

A sub cohort of *n*=10 per group was brought forward for HDL proteomic composition analysis to further probe the relationship between HDL protein composition and HDL-CEC in women vs. men.

Clinical and biochemical characteristics and HDL-CEC parameters of the sub-cohort of T1DM, T2DM and their respective age-, and BMI-matched non-diabetic participants for women and men are shown in **Table 5.4**. The medication use of the sub-cohort is shown in **Table 5.10** in **Appendix 5A**.

Table 5.4 *Baseline characteristics of sub cohort of women and men with T1DM and T2DM and their respective controls*

Characteristics	Non-OB (*n*=10)	T1DM (*n*=10)	OB (*n*=10)	T2DM (*n*=10)	Non-OB (*n*=10)	T1DM (*n*=10)	OB (*n*=10)	T2DM (*n*=10)
	Women				**Men**			
Clinical/biochemical								
Age (years)	43.5 ± 7.5	43.2 ± 6.8	42.1 ± 7.4	43 ± 6.5	42.8 ± 6.8	42.9 ± 6.8	44 ± 7.7	43.7 ± 6.3
Body mass index (kg/m^2)	24.6 ± 3.3	24.5 ± 3.1	35.8 ± 3.7	35.7 ± 4.5	25.5 ± 2.1	25.6 ± 2.3	34.5 ± 2.0	34.4 ± 2.9
Waist to hip ratio	0.85 ± 0.06	0.82 ± 0.06	0.9 ± 0.08	0.92 ± 0.04*	0.93 ± 0.08	0.92 ± 0.07	1.0 ± 0.04	1.0 ± 0.04
Smokers (%)	20	40	10	10	10	30	20	20
Systolic blood pressure (mm Hg)	130 ± 9	127 ± 13	129 ± 18	119 ±18	130 ± 8	129 ± 8	139 ±9	135 ± 12
Diastolic blood pressure (mm Hg)	86 ± 10	79 ± 4	88 ± 6	73 ± 9⋈	79 ± 6	81 ± 4	84 ± 13	79 ± 8
Haemoglobin A1c (%)	5.3 ± 0.3	7.9 ± 1.1$^{\#}$	5.5 ± 0.3	6.8 ± 0.8	5.4 ± 0.2	8.4 ± 1.1$^{\#\#\#}$	5.4 ± 0.2	8.0 ± 1.8§§
Total cholesterol (mmol/l)	5.0 ± 1.2	4.5 ± 0.5	5.4 ± 0.9	3.6 ± 1.2§	5.2 ± 0.6	4.5 ± 1.0	5.7 ± 1.3	5.9 ± 3.5
Triglycerides (mmol/l)	0.93 (0.5-1.2)	1 (0.7-1.4)	1.3 (1.1-1.8)	1.2 (0.8-3.4)	0.95 (0.9-1.5)	1.1 (0.8-1.2)	2.3 (1.3-3.6)$^{\$}$	2.9 (1.5-3.7) ⋈;**
LDL cholesterol (mmol/l)	2.8 ± 1.1	1.8 ± 0.8	3.4 ± 0.8	2.0 ±0.8§§	3.2 ± 0.4	2.5 ± 0.6$^{\#\#}$	3.6 ± 1.3	1.9 ± 0.8
HDL cholesterol (mmol/l)	1.7 ± 0.5	2 ± 0.5	1.4 ± 0.5	1.1 ± 0.2*	1.4 ± 0.3	1.5 ± 0.4	1 ± 0.2$^{\$}$	0.9 ± 0.2
HDL functionality								
Total CEC (%efflux/4hr)	13.85 ± 3.1	14.34 ± 2.4	13.18 ± 4.53	10.77 ± 1.72*	11.31 ± 2.89	14.42 ± 2.7	11.59 ± 3.9	14.2 ± 2.2
ABCA1-dependent CEC (%efflux/4hr)	6.19 ± 3.15	5.4 ± 2.54	5.3 ± 3.22	3.94 ± 1.33	4.38 ±1.9	5.78 ± 1.73	4.9 ±2.59	6.44 ± 2.07
ABCA1-independent CEC (%efflux/4hr)	7.66 ± 2.26	8.95 ± 2.46	8.53 ± 2.05	7.22 ± 0.86	6.94 ± 1.49	8.64 ± 1.48	6.7 ± 1.85	7.77 ± 1.72

Data are displayed as mean±SD, median (IQR) or %
Statistical significance is presented as
$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, $^{\#\#\#\#}P$<0.0001 for Con1 vs. T1DM;
$^{\$}P$<0.05, $^{\$\$}P$<0.01, $^{\$\$\$}P$<0.001, $^{\$\$\$\$}P$<0.0001 for Con1 vs. Con2;
⋈P<0.05, ⋈⋈P<0.01, ⋈⋈⋈P<0.001, ⋈⋈⋈⋈P<0.0001 for Con1 vs. T2DM;
$^{\S}P$<0.05, $^{\S\S}P$<0.01, $^{\S\S\S}P$<0.001, $^{\S\S\S\S}P$<0.0001 for Con2 vs. T2DM; and
*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 for T1DM vs. T2DM.
Non-OB = Non-obesity, T1DM = type 1 diabetes mellitus; OB = obesity; T2DM = type 2 diabetes mellitus.

5.3.6 Modulation of the proteomic composition of L-HDL-P across groups in women vs. men

Differences in HDL proteomic composition was initially compared between men and women with all subgroups pooled to identify sex-specific differences in HDL-associated proteins on L-HDL-P. We subsequently investigated the relationship between HDL-associated proteins and HDL-CEC to interrogate whether similar proteins were responsible for mediating cholesterol efflux across both sexes.

590 valid proteins were detected on L-HDL-P after MS.

208 proteins were identified on L-HDL-P after filtering for 70% valid proteins in at least one group. 25 proteins were significantly different between women and men. Of these, eight were significantly decreased in women including phosphatidylinositol-glycan-specific phospholipase D (GPLD1), vasorin (VASN), platelet factor 4 (PF4), beta-2-glycoprotein 1 (APOH), cadherin-13 (CDH13), cartilage oligomeric matrix protein (COMP) and attractin (ATRN). By contrast, 17 proteins were significantly increased in women including the apolipoproteins ApoA-I and ApoA-II, three proteins involved in the complement system, four immunoglobulins (Ig), the pro-inflammatory protein SAA2-SAA4, and also pregnancy zone protein (PZP) (**Figure 5.4 A&B**)**.** GPLD1, ApoH and PZP reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.4 B**)**.**

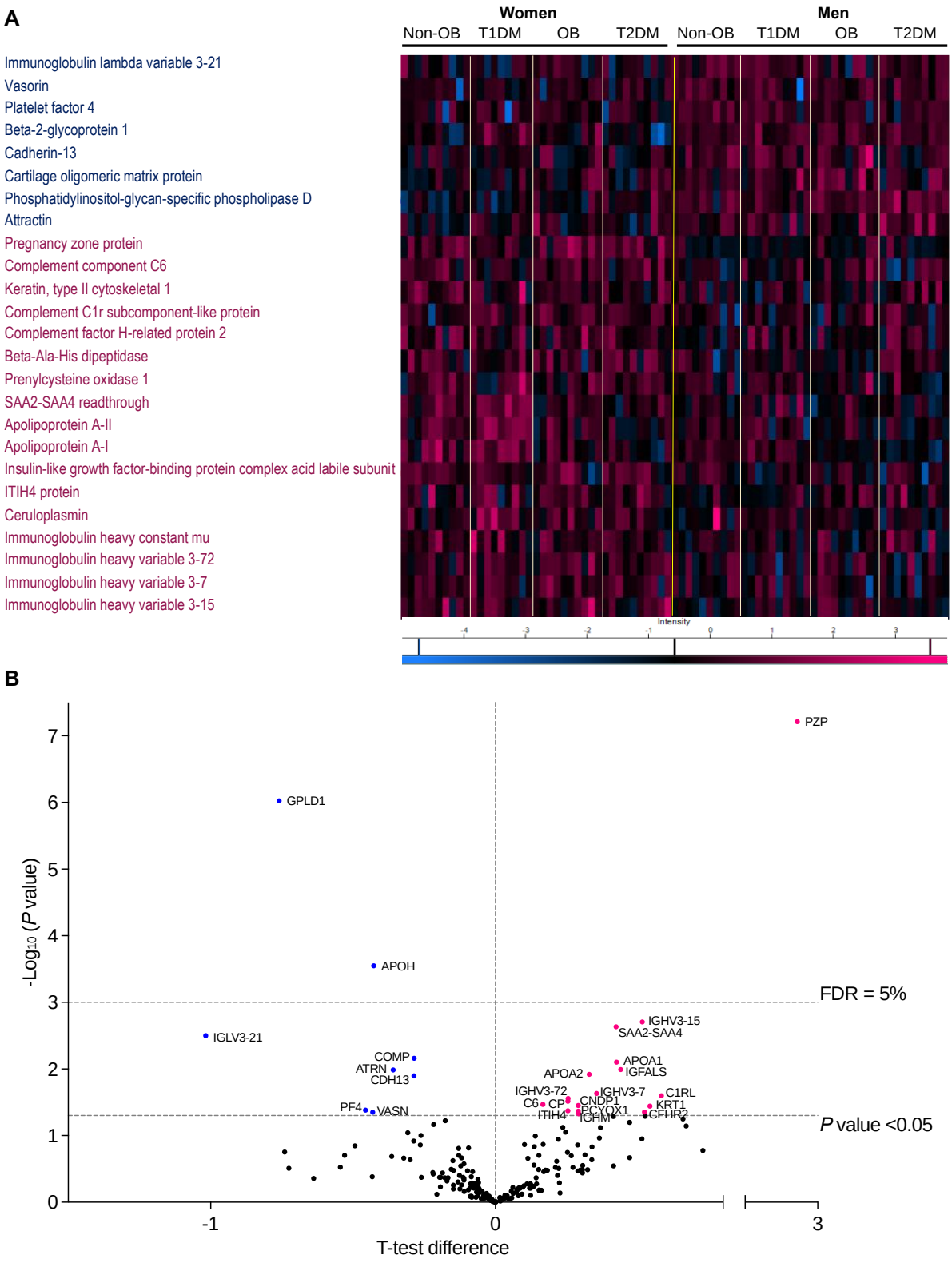

Figure 5.4 *Significantly changed proteins on large HDL-P between women and men.*
HDL was isolated via FPLC from women and men (*n*=40 per sex) with and without T1DM or T2DM (*n*=10 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between men and women were identified by mass spectrometry. **A)** Perseus software was used to determine significantly (*P*<0.05) changed proteins and presented in a heatmap.**(B)** Volcano plot showing the differences in protein abundance detected between women and men. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men. Non-OB = non-obesity; OB = Obesity.

5.3.7 Modulation of the proteomic composition of L-HDL-P in women vs. men

We subsequently sought to compare the HDL proteomic composition of L-HDL-P within the sub-groups of women and men with and without T1DM or T2DM.

590 valid proteins were detected on L-HDL-P after MS.

5.3.7.1 Modulation of the proteomic composition of L-HDL-P in women vs. men without obesity

216 proteins were identified on L-HDL-P from women and men without obesity after filtering for seven valid proteins (70%) in at least one group. 16 proteins were significantly different between women and men with BMI in the normal range. Of these, eight were significantly decreased in women including phosphatidylinositol-glycan-specific phospholipase D (GPLD1) and attractin (ATRN), proteins involved in cell migration. By contrast, eight were significantly increased in women including the apolipoprotein ApoM, the pro-inflammatory protein SAA2-SAA4, and pregnancy zone protein (PZP) (**Table *5.5*** & **Figure 5.9 A&B in Appendix 5D**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.9 B in Appendix 5D**).

5.3.7.2 Modulation of the proteomic composition of L-HDL-P in women vs. men with T1DM

211 proteins were identified on L-HDL-P in women and men with T1DM after filtering for seven valid proteins (70%) in at least one group. 12 proteins were significantly different between women and men. Of these, six were significantly decreased in women including phosphatidylinositol-glycan-specific phospholipase D (GPLD1), coagulation factor V (F5), von Willebrand factor (VWF), cadherin-13 (CDH13) and clusterin (CLU). By contrast, six were significantly increased including the apolipoproteins ApoA-I and ApoA-II, the pro-inflammatory protein SAA2-SAA4, and pregnancy zone protein (PZP) (**Table *5.5*** & **Figure 5.10 A&B in Appendix 5D**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.10 B in Appendix 5D *Table 5.5***).

5.3.7.3 Modulation of the proteomic composition of L-HDL-P in women vs. men with obesity

214 proteins were identified on L-HDL-P in women and men with obesity after filtering for seven valid proteins (70%) in at least one group. Nine proteins were significantly different between women and men with obesity. Of these, two were significantly decreased in women including carboxypeptidase N subunit 2 (CPN2) and phosphatidylinositol-glycan-specific phospholipase D (GPLD1). In contrast, seven proteins were significantly increased in women including

complement C3 (C3), angiotensinogen (AGT), three Igs, and pregnancy zone protein (PZP) (**Table *5.5*** & **Figure 5.11 A&B in Appendix 5D**).
No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.11 B in Appendix 5D**).

5.3.7.4 Modulation of the proteomic composition of L-HDL-P in women vs. men with T2DM

216 proteins were identified on L-HDL-P from women and men with T2DM after filtering for seven valid proteins (70%) in at least one group. 11 proteins were significantly different between women and men. Of these, six were significantly decreased including phosphatidylinositol-glycan-specific phospholipase D (GPLD1), attractin (ATRN) and beta-2-glycoprotein (APOH), while five were significantly increased in women including prenylcysteine oxidase 1 (PCYOX1), coagulation factor XIII B chain (F13B) and pregnancy zone protein (PZP) (**Table *5.5*** & **Figure 5.12 A&B in Appendix 5D**).
No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.12 B in Appendix 5D**).

Table 5.5 *Significantly changed proteins on L-HDL-P across groups in women vs. men.*

Non-OB	T1DM	OB	T2DM
Increased in women vs men			
Pregnancy zone protein	Pregnancy zone protein	Pregnancy zone protein	Prenylcysteine oxidase 1
Keratin, type I cytoskeletal 10	SAA2-SAA4	GDH/6PGL endoplasmic bifunctional protein	Pregnancy zone protein
Endoplasmic reticulum chaperone BiP	Cathelicidin antimicrobial peptide	Complement C3	Coagulation factor XIII B chain
Immunoglobulin J chain	Apolipoprotein A-II	Angiotensinogen	Ig-like domain-containing protein (Frag)
Complement component C6	Insulin-like growth factor-binding protein complex acid labile subunit	Probable non-functional immunoglobulin heavy variable 3-38	Immunoglobulin kappa variable 1-17
SAA2-SAA4	Apolipoprotein A-I	Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Frag)	
Apolipoprotein M		Immunoglobulin lambda variable 3-16	
Immunoglobulin heavy variable 3-15			
Decreased in women vs men			
Immunoglobulin kappa variable 1-33	Coagulation factor V	Carboxypeptidase N subunit 2	Phosphatidylinositol-glycan-specific phospholipase D
Carboxypeptidase N subunit 2	von Willebrand factor	Phosphatidylinositol-glycan-specific phospholipase D	Attractin
Beta-2-glycoprotein 1	Immunoglobulin lambda variable 3-21		Plasma protease C1 inhibitor
Cartilage oligomeric matrix protein	Cadherin-13		Kallistatin
Protein AMBP	Phosphatidylinositol-glycan-specific phospholipase D		Beta-2-glycoprotein 1
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Frag)	Clusterin		Immunoglobulin lambda variable 7-46
Phosphatidylinositol-glycan-specific phospholipase D			
Attractin			

All proteins were significant $P<0.05$.

5.3.8 Modulation of the proteomic composition of S-HDL-P across groups in women vs. men

We next determined the proteome of S-HDL-P in all groups pooled together (*n*=40 in total per group) regardless of metabolic disease. Interestingly, the proteome of smaller HDL-P is modulated to a greater extent in women relative to men, than the proteome of L-HDL-P.
590 valid proteins were detected on S-HDL-P after MS.

186 proteins were identified on S-HDL-P after filtering for 70% valid proteins in at least one group. 37 proteins were significantly different between women and men. Of these, 20 were significantly increased in women including three complement proteins; alpha-1-antichymotrypsin (SERPINA3) and alpha-1-antitrypsin (SERPINA1), proteins involved in the acute phase response; antithrombin-III (SERPINC1) which is involved in the coagulation cascade; and also two Igs. By contrast, 17 proteins were significantly decreased in women including the proinflammatory protein serum amyloid P-component, vitamin K-dependent protein S (PROS1), which is involved in blood coagulation; and bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4 (NDST4). The apolipoproteins ApoA-I, ApoC-II, ApoC-III, ApoD and ApoM were also decreased on S-HDL-P in women relative to men. This was a contrasting finding to L-HDL-P, where ApoA-I was enriched in women relative to men (**Figure 5.5 A&B**).
Five proteins reached the statistical threshold of the FDR of 5% ($-Log_{10}$=3 in volcano plot), which adjusts for multiple comparisons in large datasets. These proteins were ApoD, ApoM, ApoC-I, PROS1 and NDST4 (**Figure 5.5 B**).

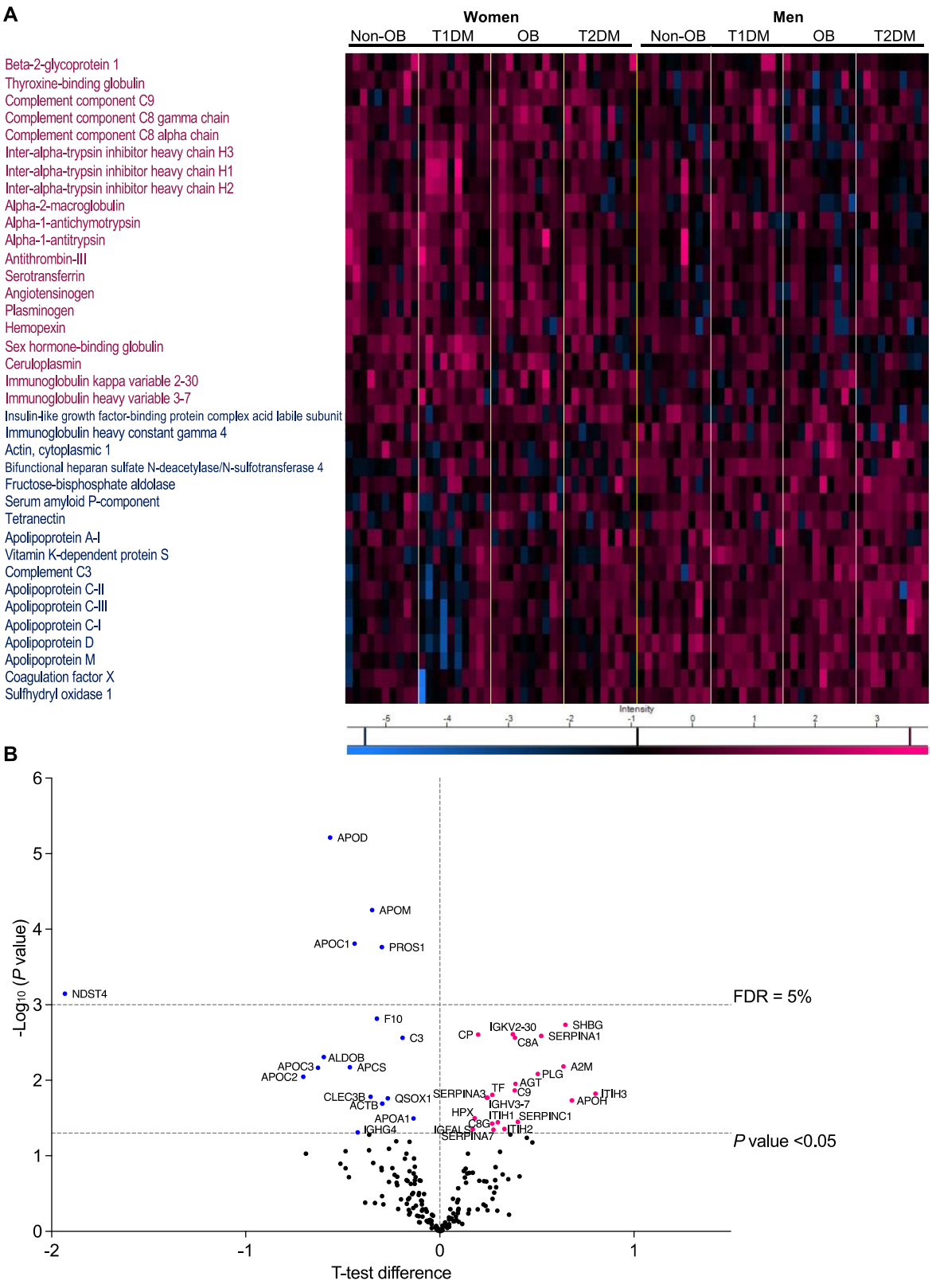

Figure 5.5 *Significantly changed proteins on small HDL-P between women and men.*
HDL was isolated via FPLC from women and men (n=40 per sex) with and without T1DM or T2DM (n=10 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between men and women were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (P<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between women and men. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a P value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men. Non-OB = non-obesity; OB = Obesity; W = women; M = men.

5.3.9 Modulation of the proteomic composition of S-HDL-P in women vs. men

We subsequently sought to compare the HDL proteomic composition of S-HDL-P within the sub-groups of women and men with and without T1DM or T2DM.

590 valid proteins were detected on S-HDL-P after MS.

5.3.9.1 Modulation of the proteomic composition of S-HDL-P in women vs. men without obesity

199 proteins were identified on S-HDL-P in women and men without obesity after filtering for seven valid proteins (70%) in at least one group. 17 proteins were significantly different between women and men. Of these, eight were significantly decreased in women including phosphatidylinositol-glycan-specific phospholipase D (GPLD1), three Igs, and also the apolipoproteins ApoD and ApoM. By contrast, nine were significantly increased in women including heparin cofactor 2 (SERPIND1) which is involved in blood coagulation; angiotensinogen (AGT) which is involved in the vascular system; and the acute phase response protein alpha-1-antichymotrypsin (SERPINA3) (**Table 5.8** & **Figure 5.13 A&B in Appendix 5E**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.13 B in Appendix 5E**).

5.3.9.2 Modulation of the proteomic composition of S-HDL-P in women vs. men with T1DM

191 proteins were identified on S-HDL-P in women and men with T1DM after filtering for seven valid proteins (70%) in at least one group. 13 proteins were significantly different between women and men. Of these, five were significantly increased in women including complement C4-B (C4B), sex-hormone binding globulin (SHBG) and two Igs. By contrast, eight were significantly decreased including the apolipoproteins ApoC-IV and ApoC-II, and ApoD; and the pro-inflammatory protein serum amyloid P-component (APCS) (**Table 5.8** & **Figure 5.14 A&B in Appendix 5E**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.14 B in Appendix 5E**).

5.3.9.3 Modulation of the proteomic composition of S-HDL-P in women vs. men with obesity

191 proteins were identified on S-HDL-P in women and men with obesity after filtering for seven valid proteins (70%) in at least one group. 13 proteins were significantly different between women and men. Of these, four were significantly decreased in women including ApoD, complement C3 (C3) and fructose-bisphosphate aldolase (ALDOB). In contrast, nine proteins were significantly increased in women including the apolipoprotein ApoL1, three

complement proteins; alpha-1-antitrypsin (SERPINA1) which is involved in the acute phase response and also antithrombin-III (SERPINC1) (**Table 5.8** & **Figure 5.15 A&B in Appendix 5E**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.15 B in Appendix 5E**).

5.3.9.4 Modulation of the proteomic composition of S-HDL-P in women vs. men with T2DM

193 proteins were identified on S-HDL-P in women and men with T2DM after filtering for seven valid proteins (70%) in at least one group. 15 proteins were significantly different between women and men. Of these, five were significantly increased in women including beta-2 glycoprotein 1 (APOH), insulin like growth factor-binding protein complex acid labile subunit (IGFALS) and three Igs. By contrast, ten were significantly decreased including serum amyloid P-component (APCS); complement C3 (C3); ApoC-I and ApoM (**Table 5.8** & **Figure 5.16 A&B in Appendix 5E**).

No protein reached the statistical threshold of the FDR of 5% ($-Log_{10}=3$ in volcano plot), which adjusts for multiple comparisons in large datasets (**Figure 5.16 A&B in Appendix 5E**).

Table 5.6 *Significantly changed proteins on S-HDL-P across groups in women vs. men.*

Non-OB	T1DM	OB	T2DM
Increased in women vs men			
Ceruloplasmin	Sex hormone-binding globulin	Alpha-2-macroglobulin	Beta-2-glycoprotein 1
A-kinase anchor protein 9	Immunoglobulin kappa variable 2-30	Complement component C8 gamma chain	Insulin-like growth factor-binding protein complex acid labile subunit
Complement component C8 alpha chain	Inter-alpha-trypsin inhibitor heavy chain H2	Complement component C8 alpha chain	Immunoglobulin heavy variable 3-7
Fibrinogen alpha chain	Complement C4-B	Complement component C9	Immunoglobulin kappa variable 2-30
Heparin cofactor 2	Immunoglobulin kappa variable 2-24	Alpha-1-antitrypsin	Immunoglobulin heavy variable 3-13
Angiotensinogen		Antithrombin-III	
Alpha-1-antichymotrypsin		Plasminogen	
Glutathione peroxidase		Alpha-1-acid glycoprotein 1	
Immunoglobulin heavy variable 3-74		Apolipoprotein L1	
Decreased in women vs men			
Phosphatidylinositol-glycan-specific phospholipase D	Complement C1s subcomponent	Apolipoprotein D	Serum amyloid P-component
Immunoglobulin heavy constant alpha 1	Apolipoprotein C-IV	Complement C3	Coagulation factor IX
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Frag)	Apolipoprotein C-II	Vitamin K-dependent protein S	Complement factor H-related protein 2
Immunoglobulin lambda variable 3-21	Apolipoprotein D	Fructose-bisphosphate aldolase	Vitamin K-dependent protein S
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	Apolipoprotein C-I		Apolipoprotein C-I
Vitamin K-dependent protein S	Coagulation factor IX		Complement component C7
Apolipoprotein D	Serum amyloid P-component		Complement C3
Apolipoprotein M	Immunoglobulin lambda variable 3-16		Tetranectin
			Plasma kallikrein
			Apolipoprotein M

All proteins were significant $P<0.05$.

5.3.10 Proteins on large and small HDL-P that were modulated between women and men across the groups

Given the different patterns of protein expression between women and men in L-HDL-P versus S-HDL-P, we graphed a number of key proteins to cross-compare with trends in the opposing particle size (**Figure 5.6** and **Figure 5.7**). Of note, the abundance of ApoA-I and ApoA-II was similar across L-HDL-P and S-HDL-P, but a specific enrichment was evident on L-HDL-P in women with T1DM relative to men (**Figure 5.6 A&B**). Pregnancy zone protein was only identified on L-HDL-P and was enriched in women relative to men (**Figure 5.6 D**). Another key protein that was consistently reduced on L-HDL-P in women across all groups was phosphatidylinositolglycan-specific phospholipase D **(Figure 5.6 E**). Similar to pregnancy zone protein, levels of this protein were extremely low/ undetectable on S-HDL-P. In contrast, beta-2-glycoprotein 1 (ApoH) had lower abundance on L-HDL-P, however it was enriched on L-HDL-P in non-obese women and women with T2DM, relative to men (**Figure 5.6 F**).

ApoM on L-HDL-P was significantly increased in non-obese women relative to men. By contrast, a significant reduction in ApoM on S-HDL-P was evident in non-obese women and also in women with T2DM relative to men – levels of HDL-ApoM however were much lower on S-HDL-P relative to L-HDL-P (**Figure 5.7 A**). A consistent finding on S-HDL-P was the enrichment of ApoD in men versus women, an observation that was not evident on L-HDL-P (**Figure 5.7 B**). This enrichment of ApoD on S-HDL-P was not evident in men with T2DM relative to women. ApoC-I was enriched on S-HDL-P in men with T1DM and T2DM relative to women counterparts (**Figure 5.7 C**). By contrast, vitamin K-dependent protein S was enriched on S-HDL-P in men without diabetes (non-obese and obese) relative to women in the same groups, with no difference between men and women with T1DM or T2DM (**Figure 5.7 D**). S-HDL-P were enriched with complement C3 relative to L-HDL-P, with significantly reduced levels evident in women with obesity and T2DM relative to men (**Figure 5.7 E**). S-HDL-P were also enriched with serum amyloid component P, with a significant reduction evident in women with T2DM relative to men with T2DM (**Figure 5.7 F**).

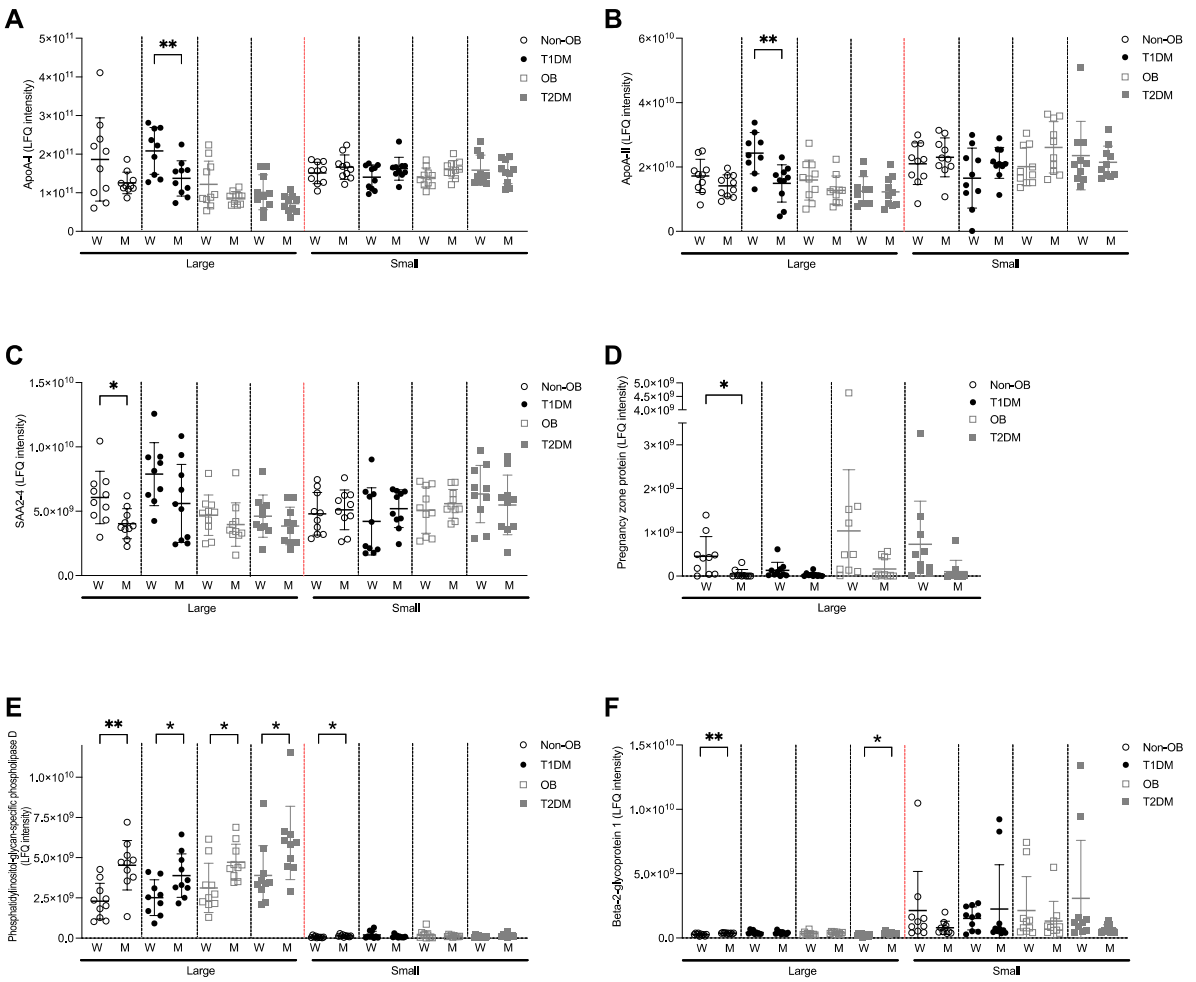

Figure 5.6 *Significantly changed proteins on HDL-P between women and men.*
HDL was isolated via FPLC from women and men with/ without T1DM or T2DM (*n*=10 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) and small HDL-P (fractions 40-42) between men and women were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** ApoA-I, **(B)** ApoA-II **(C)** SAA2-SAA4**, (D)** Pregnancy zone protein, **(E)** Phosphatidylinositolglycan-specific phospholipase D, and **(F)** Beta-2-glycoprotein 1. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for women vs. men.
Non-OB = non-obesity; OB = Obesity; W = women; M = men.

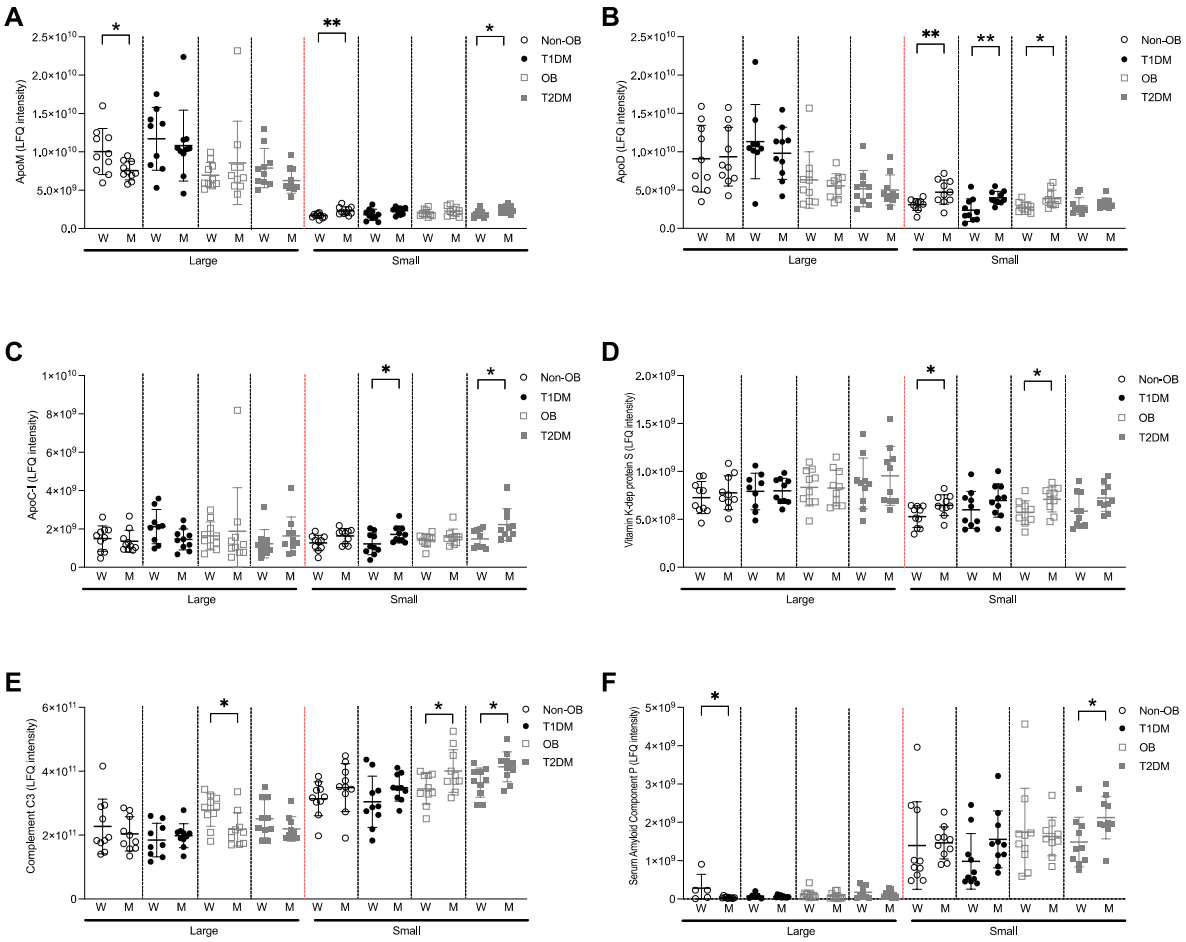

Figure 5.7 *Significantly changed proteins on HDL-P between women and men.*
HDL was isolated via FPLC from women and men with and without T1DM or T2DM (*n*=10 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) and small HDL-P (fractions 40-42) between men and women were identified by mass spectrometry. Dot plots show raw LFQ intensity results for **(A)** ApoM, **(B)** ApoD, **(C)** ApoC-I, **(D)** Vitamin K-dependent protein S, **(E)** Complement C3, and **(F)** Serum amyloid component P. Data are displayed as mean ± SD. Statistical significance is presented as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 for women vs. men.
Non-OB = non-obesity; OB = Obesity; W = women; M = men.

5.3.11 Associations between the proteomic composition of L-HDL-P and HDL-CEC parameters in women and men

We next sought to investigate the correlation between HDL-associated proteins and HDL-CEC in women and men and identify potential protein biomarkers of HDL-CEC that may be unique to women and men. We combined all groups of each sex into one group (*n*=40 per sex) to increase the *n* numbers for this analysis as an *n*=10 per group wouldn't be powered to reveal protein biomarkers of HDL-CEC across the groups. However, this analysis for both the L-HDL-P and S-HDL-P within the sub-groups is presented in **Appendix 5F & Appendix 5G.**

Looking at the correlations with all groups combined, there were different proteins on L-HDL-P that correlated with HDL-CEC parameters in women and men, some of which had opposing effects **Table 5.7**.

ApoA-II on L-HDL-P significantly positively correlated with total CEC to a greater extent in women (r=0.574) than in men (r=0.328). ApoC-I, ApoC-III and ApoD on L-HDL-P had moderate significant positive correlations with total CEC in women only, while ApoM on L-HDL-P had a positive correlation in men only. ApoA-II on L-HDL-P was the only apolipoprotein in women to have a significant positive correlation with ABCA1-dependent CEC; in men ApoE on L-HDL-P positively associated with ABCA1-dependent CEC. The majority of apolipoproteins on L-HDL-P significantly positively correlated with ABCA1-independent CEC in women including ApoA-I, ApoA-II, ApoC-I, ApoC-IV, ApoD, ApoE, ApoF and ApoM. Associations between apolipoproteins on L-HDL-P and ABCA1-independent CEC were much weaker in men with significant associations evident to ApoM and ApoC-II only.

The HDL remodelling protein, phosphatidylcholine-sterol acyltransferase (LCAT), on L-HDL-P significantly positively correlated with total CEC and ABCA1-dependent CEC in men only. In women, LCAT significantly positively correlated with ABCA1-independent CEC only. The pro-inflammatory protein SAA2-4 significantly positively associated with total CEC and ABCA1-independent CEC in both women and men.

Interestingly, the majority of Igs on L-HDL-P were negatively correlated with all three HDL-CEC parameters in men. In contrast, the majority of Igs on L-HDL-P were positively correlated with total CEC and ABCA1-dependent CEC in women. The acute phase protein, inter-alpha-trypsin inhibitor heavy chain H 4 (ITIH4) was associated with total CEC in men and women, with a greater association to ABCA1-dependent CEC in women (r=0.413) than in men (r=0.297 ns).

Table 5.7 *Proteins on large HDL that significantly correlated with HDL-CEC parameters when groups were combined in women and men (n=40 per sex).*

L-HDL-associated protein	Total efflux		ABCA1-dep		ABCA1-indep	
	Women	Men	Women	Men	Women	Men
A-kinase anchor protein 9	0.364	-0.353	0.394*	-0.285	-0.025	-0.304
Adipocyte plasma membrane-associated protein	0.181	0.426**	-0.124	0.250	0.469**	0.470**
Alpha-2-HS-glycoprotein	0.293	0.354*	0.231	0.355*	0.142	0.209
Apolipoprotein A-I	0.315	0.088	0.052	-0.054	0.432**	0.228
Apolipoprotein A-II	0.574***	0.328*	0.332*	0.270	0.450**	0.265
Apolipoprotein C-I	0.426**	0.250	0.121	0.200	0.513***	0.211
Apolipoprotein C-II	0.197	0.245	0.116	0.101	0.150	0.323*
Apolipoprotein C-III	0.406*	0.257	0.264	0.310	0.276	0.087
Apolipoprotein C-IV	0.296	-0.136	-0.010	-0.196	0.482**	-0.008
Apolipoprotein D	0.518***	0.021	0.252	-0.156	0.474**	0.230
Apolipoprotein E	0.015	0.255	-0.209	0.395*	0.322*	-0.022
Apolipoprotein F	0.281	0.171	0.072	0.048	0.349*	0.254
Apolipoprotein M	0.288	0.337*	-0.046	0.122	0.530***	0.465**
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	0.095	−0.349*	0.070	-0.329	0.058	-0.240
C4b-binding protein alpha chain	0.111	0.399*	-0.167	0.283	0.417**	0.380*
Cadherin-13	0.424**	-0.078	0.357*	-0.145	0.171	0.036
Cadherin-5	0.040	0.328*	-0.144	0.200	0.271	0.354*
Ceruloplasmin	0.147	0.221	0.246	0.359*	-0.114	-0.039
Coagulation factor V	-0.298	0.287	-0.124	0.020	-0.314	0.502**
Coagulation factor XIII B chain	0.113	0.314*	0.017	0.288	0.156	0.219
Complement C1r subcomponent-like protein	0.253	0.144	0.375*	-0.012	-0.105	0.271
Complement C1s subcomponent	-0.301	0.065	−0.445**	-0.001	0.153	0.121
Complement C4-B	−0.363*	-0.079	−0.359*	-0.028	-0.071	-0.110
Complement component C6	−0.318*	0.137	-0.287	0.075	-0.102	0.158
Complement component C7	0.060	0.369*	0.026	0.195	0.060	0.435**
Complement component C8 alpha chain	0.414*	0.087	0.300	-0.028	0.212	0.185
Complement factor H-related protein 1	0.339*	0.179	0.258	0.001	0.177	0.328*
Fibulin-1	-0.010	−0.384*	0.039	−0.389*	-0.061	-0.216
Gelsolin	0.412**	-0.119	0.445**	-0.195	0.026	0.024
Hemopexin	-0.002	0.335*	-0.022	0.380*	0.029	0.143
Hepatocyte growth factor activator	-0.027	0.180	-0.122	-0.003	0.131	0.332*
Hyaluronan-binding protein 2	0.520**	-0.232	0.525**	-0.159	0.079	-0.211
Ig-like domain-containing protein (Fragment)	0.350*	-0.045	0.286	-0.128	0.160	0.081
Ig-like domain-containing protein (Fragment)	0.168	−0.426**	0.307	-0.294	-0.168	−0.416**
Immunoglobulin heavy constant alpha 1	0.033	-0.258	0.100	-0.120	-0.090	−0.324*
Immunoglobulin heavy constant alpha 2 (Fragment)	0.433*	0.120	0.219	0.044	0.384*	0.170
Immunoglobulin heavy constant gamma 3	0.149	−0.578****	0.031	−0.496**	0.196	−0.446**
Immunoglobulin heavy variable 3-7	0.187	-0.280	0.342*	-0.213	-0.189	-0.249
Immunoglobulin heavy variable 3-72	0.135	−0.317*	0.117	-0.229	0.049	-0.297
Immunoglobulin heavy variable 3-73	0.236	-0.054	0.343*	-0.132	-0.110	0.068
Immunoglobulin heavy variable 3-9	0.017	0.116	0.250	0.116	−0.329*	0.067
Immunoglobulin heavy variable 4-38-2	0.204	-0.156	0.329*	-0.237	-0.145	0.012
Immunoglobulin kappa variable 1-33	0.224	−0.334*	0.332*	-0.299	-0.115	-0.241
Immunoglobulin kappa variable 2D-29	0.351*	−0.347*	0.173	−0.365*	0.306	-0.187
Immunoglobulin kappa variable 6-21	0.047	-0.303	0.121	−0.322*	-0.098	-0.157
Immunoglobulin lambda constant 3	0.017	−0.398*	0.076	-0.253	-0.082	−0.416**
Immunoglobulin lambda variable 1-47	0.298	-0.253	0.374*	-0.292	-0.066	-0.096
Immunoglobulin lambda variable 3-10	0.122	−0.400*	0.183	−0.426**	-0.064	-0.206
Immunoglobulin lambda variable 3-21	-0.263	−0.458**	-0.334	−0.396*	0.054	−0.384*
Immunoglobulin lambda variable 3-9	0.044	−0.372*	0.098	−0.331*	-0.070	-0.271
Immunoglobulin lambda variable 5-45	0.008	−0.322*	0.013	-0.224	-0.005	-0.313
Immunoglobulin lambda variable 8-61	0.448**	0.140	0.465**	-0.020	0.060	0.281
Insulin-like growth factor-binding protein 3	-0.214	0.247	-0.223	0.025	-0.023	0.409*
Interleukin-1 receptor accessory protein	0.272	0.234	0.360*	0.066	-0.082	0.357*
ITIH4 protein	0.322*	0.337*	0.413**	0.297	-0.073	0.249
Phosphatidylcholine-sterol acyltransferase	0.151	0.405**	-0.103	0.359*	0.391*	0.297
Prenylcysteine oxidase 1	0.204	0.213	-0.062	-0.040	0.416**	0.438**
SAA2-SAA4	0.409**	0.324*	0.062	0.154	0.570***	0.403**
Serum paraoxonase/lactonase 3	0.262	0.078	0.070	-0.166	0.316	0.348*
Transthyretin	0.301	0.005	0.364*	0.186	-0.037	-0.220
Vasorin	-0.183	-0.268	-0.026	−0.347*	-0.252	-0.059
Vitronectin	-0.146	-0.063	-0.051	0.203	-0.162	−0.366*

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

5.3.12 Associations between the proteomic composition of S-HDL-P and HDL-CEC parameters in women and men

We further investigated the relationship between the proteome of S-HDL-P and HDL-CEC in men versus women **Table 5.8**.

No apolipoprotein on S-HDL-P significantly correlated with any of the HDL-CEC parameters in women. In men, ApoA-II negatively correlated and ApoC-I positively correlated with total CEC; ApoD negatively correlated, while ApoC-I, ApoC-III and ApoE positively correlated with ABCA1-dependent CEC and ApoA-IV positively correlated with ABCA1-independent CEC.

In men, the coagulation factor proteins V, IX and X on S-HDL-P significantly positively associated with total CEC; Coagulation factor V correlated with ABCA1-independent CEC while factor IX and X positively correlated with ABCA1-dependent CEC. In contrast, in women coagulation factor IX negatively associated with total CEC, and no relationship was evident with factor V or X to any HDL-CEC parameter.

The complement proteins on S-HDL-P, C3/C5 convertase, C4b-binding protein alpha chain, complement C2, complement component C8 alpha chain, beta chain and gamma chain, and complement component C9 positively associated with both total CEC and ABCA1-independent CEC in men only. By contrast, complement component C8 gamma chain negatively correlated with total CEC, as well as ABCA1-dependent CEC in women. Additionally, complement C1r subcomponent-like protein and complement C2 negatively associated with ABCA1-dependent CEC in women only, with otherwise little relationship between complement proteins on S-HDL-P and HDL-CEC in women.

Similarly to the Igs on L-HDL-P, the majority of Igs on S-HDL-P in women were positively associated with HDL-CEC. In men the majority of Igs were negatively associated with HDL-CEC, however, there were few Igs that were significant.

LCAT on S-HDL-P negatively associated with total CEC and ABCA1-dependent CEC in men only.

Table 5.8 *Proteins on small HDL that significantly correlated with HDL-CEC parameters when groups were combined in women and men (n=40 per sex).*

S-HDL-associated protein	Total efflux		ABCA1-dep		ABCA1-indep	
	Women	Men	Women	Men	Women	Men
Alpha-1-acid glycoprotein 1	−0.372*	0.219	-0.196	0.223	-0.274	0.154
Alpha-1-antichymotrypsin	0.104	0.345*	0.179	0.165	-0.089	0.427**
Alpha-1B-glycoprotein	0.033	0.342*	-0.166	0.363*	0.290	0.177
Alpha-2-antiplasmin	-0.210	0.169	−0.385*	0.115	0.211	0.168
Apolipoprotein A-II	0.174	−0.332*	0.034	-0.277	0.232	-0.265
Apolipoprotein A-IV	-0.111	0.298	0.019	0.163	-0.205	0.345*
Apolipoprotein C-I	0.001	0.336*	-0.148	0.422**	0.213	0.093
Apolipoprotein C-III	0.155	0.293	0.004	0.361*	0.244	0.089
Apolipoprotein D	0.197	-0.280	0.100	−0.405**	0.173	-0.012
Apolipoprotein E	-0.196	0.189	-0.202	0.325*	-0.028	-0.055
Beta-Ala-His dipeptidase	0.219	0.220	0.062	0.443**	0.264	-0.144
C3/C5 convertase	-0.153	0.481**	-0.178	0.422**	0.008	0.359*
C4b-binding protein alpha chain	0.149	0.515***	-0.124	0.474**	0.418**	0.357*
Coagulation factor V	-0.067	0.334*	-0.128	0.195	0.070	0.363*
Coagulation factor IX	−0.331*	0.351*	-0.281	0.347*	-0.132	0.214
Coagulation factor X	0.196	0.381*	0.018	0.400*	0.290	0.203
Coagulation factor XIII B chain	0.349*	-0.170	0.251	-0.145	0.204	-0.133
Complement C1r subcomponent-like protein	−0.329*	0.026	−0.339*	-0.122	-0.051	0.199
Complement C1s subcomponent	-0.163	-0.245	-0.119	−0.385*	-0.093	0.027
Complement C2	-0.230	0.373*	−0.315*	0.297	0.079	0.317*
Complement component C8 alpha chain	-0.184	0.441**	-0.204	0.236	-0.005	0.515***
Complement component C8 beta chain	-0.149	0.391*	-0.140	0.173	-0.040	0.502***
Complement component C8 gamma chain	−0.430**	0.424**	−0.414**	0.263	-0.101	0.451**
Complement component C9	-0.018	0.453**	-0.020	0.249	-0.001	0.522***
Complement factor H-related protein 1	0.416**	0.168	0.378*	0.060	0.130	0.233
Complement factor H-related protein 2	0.304	0.017	0.332*	-0.022	0.020	0.059
Complement factor I	-0.087	0.310	0.086	0.179	-0.262	0.346*
Cysteine-rich secretory protein 3	0.079	−0.327*	0.055	-0.285	0.051	-0.246
Endoplasmic reticulum chaperone BiP	-0.011	0.328*	-0.027	0.193	0.023	0.369*
Fibulin-1	0.189	-0.051	0.368*	-0.279	-0.210	0.255
Hemopexin	−0.315*	0.055	-0.277	-0.020	-0.111	0.126
Heparin cofactor 2	-0.128	0.445**	-0.108	0.405**	-0.052	0.313*
Hyaluronan-binding protein 2	0.005	0.303	0.041	0.149	-0.051	0.377*
Immunoglobulin heavy constant gamma 3	0.130	−0.477**	0.149	−0.477**	-0.003	-0.284
Immunoglobulin heavy variable 1-46	0.166	0.046	0.331*	0.043	-0.192	0.031
Immunoglobulin heavy variable 1-69	0.327*	0.005	0.483**	-0.063	-0.163	0.086
Immunoglobulin heavy variable 3-13	0.246	-0.292	0.073	-0.146	0.292	−0.360*
Immunoglobulin heavy variable 3-7	0.311	0.008	0.324*	-0.025	0.039	0.045
Immunoglobulin heavy variable 3-72	0.444**	-0.113	0.149	-0.038	0.503***	-0.161
Immunoglobulin heavy variable 3-73	0.322*	-0.101	0.190	-0.154	0.248	0.005
Immunoglobulin heavy variable 3-74	0.250	-0.056	0.363*	-0.064	-0.113	-0.026
Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Fragment)	0.367*	-0.155	0.157	-0.173	0.376*	-0.078
Immunoglobulin heavy variable 4-38-2	0.427**	0.004	0.414**	-0.058	0.102	0.080
Immunoglobulin heavy variable 5-51	0.323*	-0.013	0.354*	-0.054	0.016	0.044
Immunoglobulin heavy variable 6-1	0.413**	0.169	0.261	0.207	0.298	0.053
Immunoglobulin kappa variable 2-30	0.182	-0.094	-0.022	-0.125	0.324*	-0.019
Immunoglobulin kappa variable 2D-29	0.352*	-0.241	0.122	-0.187	0.436**	-0.209
Immunoglobulin kappa variable 3-20	0.246	-0.105	0.017	-0.046	0.371*	-0.135
Immunoglobulin kappa variable 6-21	0.228	-0.032	0.027	-0.068	0.331*	0.025
Immunoglobulin lambda constant 3	0.170	−0.411**	0.163	-0.247	0.041	−0.446**
Immunoglobulin lambda variable 5-45 (Fragment)	-0.053	−0.330*	-0.119	-0.177	0.085	−0.383*
Immunoglobulin lambda variable 7-46	0.258	-0.286	0.031	-0.294	0.377*	-0.166
Immunoglobulin lambda variable 8-61	0.391*	0.328*	0.274	0.139	0.243	0.427**
Immunoglobulin lambda-like polypeptide 5	0.048	−0.330*	0.018	-0.273	0.052	-0.267
Insulin-like growth factor-binding protein complex acid labile subunit	-0.286	0.142	−0.354*	0.192	0.045	0.023
Inter-alpha-trypsin inhibitor heavy chain H3	-0.008	0.188	0.157	-0.014	-0.238	0.360*
Keratin, type I cytoskeletal 10	0.191	-0.011	-0.118	-0.134	0.506**	0.141
Keratin, type II cytoskeletal 1	0.277	0.185	0.120	0.014	0.274	0.320*
Kininogen-1	0.227	0.412**	0.179	0.411**	0.111	0.246
L-lactate dehydrogenase B chain	0.313	-0.108	0.057	0.008	0.375*	-0.206
L-selectin	0.155	−0.359*	0.270	−0.400	-0.132	-0.158
Phosphatidylcholine-sterol acyltransferase	0.127	−0.357*	0.204	-0.265	-0.092	−0.325*
Plasminogen	-0.056	0.306	0.011	0.185	-0.106	0.331*
Platelet factor 4	0.092	0.299	-0.117	0.287	0.332*	0.191
Probable non-functional immunoglobulin heavy variable 3-35	0.341	−0.392*	0.238	−0.380*	0.239	-0.280
Probable non-functional immunoglobulin heavy variable 3-38	0.334*	0.130	0.229	0.108	0.215	0.109
Protein Z-dependent protease inhibitor	-0.012	0.260	-0.088	0.132	0.106	0.313*
Serum amyloid P-component	-0.031	0.222	0.038	0.350*	-0.104	-0.026
Vitamin K-dependent protein C	0.133	0.372*	0.265	0.372*	-0.164	0.222

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

5.4 Discussion

This study took a comprehensive approach to understanding differences in HDL-P composition and function in women relative to men with and without T1DM or T2DM. We demonstrate important differences in HDL-P size, function and composition in women relative to men, that would otherwise not be captured in static measurements of HDL-C. We demonstrate that non-obese women exhibit increased total CEC relative to men; this beneficial effect of the female sex is negated in women with T1DM and in women with obesity with equivalent HDL-CEC evident compared to men. By contrast, women with T2DM exhibit significantly reduced total CEC, and in particular ABCA1-dependent CEC, relative to men with T2DM, indicative of not only a negation of the beneficial effect of female sex on HDL-biology but a preponderance to increased CVD risk relative to men, despite equivalent HDL-C levels. The concentration of L-HDL-P, HDL-P size and HDL-C, but not S-HDL-P concentration, were important determinants of HDL-CEC in both sexes. In turn, women in the non-obese and T1DM groups exhibited significantly higher concentrations of L-HDL-P relative to men; this potential beneficial HDL-P size profile in women is negated in obesity and T2DM where no difference in the concentration of L-HDL-P was evident between sexes. The concentration of S-HDL-P was higher in men relative to women across most groups, with the exception of women and men with obesity. The HDL proteome of both L-HDL-P and S-HDL-P was significantly modulated between men and women, and in turn the proteins governing HDL-CEC were significantly different between the sexes. In particular, it was notable that apolipoproteins were more dominant drivers of CEC in women compared to men, while complement proteins were positive drivers of CEC in men compared to women. Of interest, association of Igs on HDL were negatively associated with CEC in men but positively associated in women. Our findings therefore show important sex-specific differences in the proteins governing HDL-CEC in women versus men that may have important implications for their respective cardiovascular risk profile. Indeed, our findings indicate the use of separate biomarkers to predict HDL dysfunction in men and women.

The size of HDL-P is important in accessing their anti-atherogenic properties [31]. Capturing measures of particle size, in turn, gives even greater insight into the cardiovascular risk profile of a given individual. Women without metabolic disease tend to have a more favourable lipoprotein-lipid profile compared to men, with more L-HDL-P, potentially accounting for their lower CVD risk [16]. Consistent with these findings we demonstrate an increase in L-HDL-P concentration, reduction in S-HDL-P concentration and overall increase in HDL diameter, in non-obese women and women with T1DM relative to men within the same groups. This difference in HDL-P size profile, coupled with increased total CEC, in non-obese women compared to non-obese men, may contribute to cardioprotective benefits of female sex in the absence of metabolic disease. In men with T1DM, consistent with an increase in L-HDL-P size,

there is an increase in HDL-CEC compared to their respective BMI-matched controls. By contrast, in women with T1DM, the more favourable HDL profile does not translate into significantly enhanced HDL-CEC. In our previous published paper, we showed that total CEC was significantly increased in men and, to a lesser extent in women with T1DM compared to their respective controls. This was concurrent with a profound increase in ABCA1-independent CEC evident in men but not women with T1DM relative to their controls [27]. In this study, we see similar significant increases in total CEC and ABCA1-independent CEC in men, while no differences were observed in women between T1DM and their controls probably due to lower *n* numbers (*n*=60/40 women/men published study vs. *n*=20/20 in this study). We found no differences in HDL-CEC between women and men with obesity, or relative to their non-obese sex-matched control. However, we observed a significant reduction in total CEC and ABCA1-dependent CEC in women with T2DM compared to men with T2DM, and with respect to both non-obese and obese sex-matched control groups, which could underlie the increased risk of CVD in women with T2DM.

We next investigated the relationship between HDL-P size and efflux capacity in men versus women, with and without T1DM or T2DM, and in all groups combined, to gain a greater understanding of the governance of HDL-CEC in both sexes. We demonstrated that L-HDL-P concentration, HDL-P diameter and HDL-C are the dominant drivers of total CEC and ABCA1-independent CEC, but not ABCA1-dependent CEC in both men and women. These findings are consistent with an important role for L-HDL-P in facilitating ABCA1-independent efflux [23]. Weak associations between HDL-C and HDL size and ABCA1-dependent CEC were evident in women, only when all groups were combined, with no such relationships evident in men. In women with obesity there was a strong correlation between HDL-C and ABCA1-dependent CEC (r=0.614), an effect that was not evident in men (r=−0.083). HDL-C also strongly correlated with total CEC in women with obesity (r=0.774) but not men with obesity (r=0.019); this correlation was profoundly reduced in T2DM (r=0.117 in women and r=0.074 in men).
By contrast, total HDL-P concentration strongly correlated with ABCA1-dependent CEC in men with obesity (r=0.660) but not in women with obesity (r=0.114); again this association was lost in the setting of T2DM. Interestingly, total HDL-P concentration was a better predictor of total CEC in women with T2DM (r=0.593), and to a lesser extent men (r=0.390, non-significant), than HDL-C (r=0.177 and r=0.074 respectively). These findings highlight the complex relationships between HDL-P size and function that differ dependent on sex and metabolic disease status. In certain sub-groups (e.g., women with obesity), HDL-C is a superior biomarker of HDL function, while in other sub-groups (men with obesity/ women with T2DM), HDL-P size may proffer better CVD risk assessment.

The concentration of S-HDL-P did not correlate with any HDL-CEC parameters when all groups were combined, or with total CEC in any sub-group and only positively with ABCA1-dependent CEC in women with T2DM, and negatively with ABCA1-independently in women with obesity. It is noteworthy however, that the presence of preβ-1 HDL-P, the dominant mediator of ABCA1-dependent CEC [32], is not captured in measures of NMR [33], and thus the S-HDL-P captured may not represent the dominant acceptor via ABCA1.

We subsequently investigated differences in HDL-P protein composition between men and women with and without T1DM or T2DM. It is notable that a relatively low number of proteins are different between the two sexes with greater modulation of proteins evident on S-HDL-P (37/186 proteins) compared to L-HDL-P (25/208). Furthermore, when all sub-groups were combined into one group (*n*=40), only three proteins on L-HDL-P (GPLD1, ApoH and PZP) and five proteins on S-HDL-P (ApoD, ApoM, ApoC-I, NDST4 and PROS1) reached the threshold for significance upon correction for multiple comparisons. No proteins reached the FDR cut-off when groups were sub-divided according to metabolic disease status which was likely attributable to low *n*-numbers within the sub-groups.

L-HDL-P from women were enriched with pregnancy zone protein (PZP), ApoA-I, ApoA-II and SAA2-4 and depleted of phosphatidylinositol-glycan specific phospholipase D (GPLD1) and beta-2-glycoprotein 1 (APOH) relative to men. By contrast, ApoA-I, ApoC-I, ApoC-II, ApoC-III, ApoD, ApoM, vitamin K-dependent protein S (PROS1) and bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4 (NDST4) were depleted, while complement component C8 (gamma chain and alpha chain), sex hormone binding globulin (SHBG) and alpha-1-antitrypsin were enriched on S-HDL-P in women relative to men. PZP, which is dramatically upregulated in pregnancy, hence the name, efficiently inhibits the aggregation of misfolded proteins, including the amyloid beta peptide, which forms plaques in preeclampsia and in Alzheimer's disease [34]. It was consistently enriched on L-HDL-P in women across all the groups separated. However, PZP was not correlated to any of the HDL-CEC parameters and is probably more of a biomarker of the female sex. Enrichment of L-HDL-P with SAA2-4 in women compared to men potentially indicates a shift towards a pro-inflammatory L-HDL-P. We have previously reported enrichment of HDL with SAA in people with T1DM [35], and presence of SAA is thought to displace ApoA-I on HDL-P [36], particularly during the acute phase response [37]. However, in our study, ApoA-I and SAA2-4 were both increased on L-HDL-P in women with T1DM compared to men, highlighting that the level of SAA enrichment in women with T1DM was not sufficient to displace ApoA-I. We also demonstrated a positive association between HDL-associated SAA2-4 on L-HDL-P and total CEC and also ABCA1-independent CEC in all groups combined. SAA is a known acceptor of cellular cholesterol *in vitro* [36], and

could potentially be responsible for supporting HDL-CEC particularly via ABCA1-independent pathways. By contrast, enrichment of S-HDL-P with complement C3 in men compared to women, particularly in metabolic disease groups, concurrent with an enrichment of serum amyloid component P (ACPS) in men with T2DM compared to women with T2DM, indicates a shift towards a pro-inflammatory S-HDL-P in men with metabolic disease. Alwaili *et al.,* found increased abundance of SAA, and complement C3 on the HDL proteome in men with acute coronary syndromes, reflecting a shift towards a proinflammatory particle which might alter the protective effects of HDL on the atherosclerotic plaque [38]. While complement C3 had no association to any HDL-CEC parameter within this study, we have previously found a negative correlation between complement C3 on L-HDL-P and ABCA1-independent CEC in non-obese control groups, and a positive association between complement C3 on S-HDL-P and total CEC in people with T2DM (Chapter 4). Serum amyloid complement P (ACPS), which was enriched on S-HDL-P in men, was positively correlated with ABCA1-dependent CEC in men. ACPS exists in human atherosclerotic plaques and presence is associated with CVD severity. HDL-P are thought to be an important source of ACPS in atherosclerotic lesions and ACPS-associated with HDL promotes SR-B1 dependent cholesterol efflux [39]. Whether HDL-associated ACPS in men contributes to increased CVD pathogenesis by depositing ACPS in atherosclerotic lesions or whether the accumulation of ACPS on HDL in men is net cardioprotective by supporting CEC is unknown and warrants further interrogation. Our findings point to an important role for HDL-associated ACPS in mediating ABCA1-dependent CEC in men, indicative of a compensatory mechanism at play in atherosclerotic lesions, to reduce lesion burden.

GPLD1 was consistently depleted on L-HDL-P in women across all the groups and also on S-HDL-P in women without obesity. GPLD1 specifically hydrolyses the glycan-phosphatidylinositol linkages of GPI-anchor proteins and is assumed to play a key regulatory role for the activity of these proteins [40]. Cardner *et al.,* reported that GPLD1 improves the activity of reconstituted HDL (rHDL) to inhibit the apoptosis of HAECs and that a neutralising anti-GPLD1 antibody decreased the antiapoptotic activity of both native HDL and GPLD1 containing rHDL [40]. Within our study, GPLD1 was not significantly associated with any of the HDL-CEC parameters, indicating no role in governing HDL-CEC functionality. The anti-apoptotic functions of HDL were not assessed in our study but our findings suggest greater potential of HDL from men to mediate such effects, relative to women, based on GPLD1-association with the particles, which warrants further interrogation. Another protein depleted in women, and specifically on L-HDL-P in women without obesity and also women with T2DM was ApoH. ApoH has a role in triglyceride-rich lipoprotein clearance [41] as well as being involved in insulin resistance [42]. Castro *et al.,* found that ApoH was elevated in the plasma

and liver of people with T2DM and metabolic syndrome [43]. Within these previous studies, men and women were investigated collectively and our study would highlight that ApoH has differential abundance levels in women and men. Enrichment of ApoH on HDL-P in men with T2DM coincides with increased systemic triglyceride levels in men with T2DM relative to women with T2DM; one may therefore posit that increased ApoH on HDL in men provides ApoH to TG-rich lipoproteins to aid TG-clearance from circulation [44]. ApoH was not significantly associated with any of the HDL-CEC parameters, however, in the previous chapter, ApoH on L-HDL-P was significantly correlated with total and ABCA1-dependent CEC in T2DM only, indicating that it may play a role in efflux, in particular, in T2DM.

Vitamin K-Dependent Protein S (PROS1) on S-HDL-P was also depleted in women relative to men across all groups, with the exception of T1DM. PROS1 is an anticoagulant protein and depletion in women relative to men may predispose to increased coagulability and risk of venous thrombosis [45]. PROS1 had no association to any HDL-CEC parameter within this study.

Despite enrichment of ApoM on S-HDL-P in men, ApoM on S-HDL-P was not significantly associated with any HDL-CEC parameter. Indeed, ApoM was more lowly abundant on S-HDL-P compared to L-HDL-P. Yao *et al.,* have demonstrated that ApoM-enriched HDL mediates greater cholesterol efflux from murine macrophages than ApoM-free HDL, indicative of a functional role for ApoM in driving cellular CEC [46]. ApoM on L-HDL-P was positively associated with ABCA1-independent CEC in men and women and was one of only two apolipoproteins associated with efflux in men. These findings indicate that ApoM may have an important role for supporting HDL-CEC in both sexes. ApoC-I and ApoD were depleted on S-HDL-P in women relative to men. ApoC-I plays an important role in HDL/ VLDL remodelling, whereby it slows the clearance of triglyceride-rich lipoproteins [47]. De Hann *et al.,* reported that enrichment of HDL with ApoC-I dose-dependently decreased HDL cholesterol ester association with SR-B1, in mice [48], which may impact the latter stages of cholesterol transport in men due to enrichment of HDL with ApoC-I. By contrast, Westerterp *et al.,* demonstrated that ApoC-I can support total cholesterol efflux from macrophages *in vitro* to a similar extent as ApoA-I [49]. Our data similarly supports a potential role for ApoC-I in mediating CEC in both men and women. However, our data suggests that ApoC-I on L-HDL-P is a positive determinant of total and ABCA1-independent CEC in women, but not men, while ApoC-I on S-HDL-P was positively associated with total and ABCA1-dependent CEC in men but not women. These findings indicate sex-specific differences associated with the role of ApoC-I in CEC that warrants further investigation.

ApoD similarly exhibits differential association with HDL-CEC between the sexes. ApoD on L-HDL-P positively associated with total and ABCA1-independent in women, but not men.

However, in men ApoD on S-HDL-P negatively correlated with ABCA1-dependent CEC, with no such association evident in women. In contrast to our findings a previous study by Pamir *et al.,* demonstrated that ABCA1-specific efflux correlated strongly with ApoD in a mouse model, demonstrating potential discrepancies in association between ApoD and CEC between mice and humans [50]. While the role of ApoD in supporting cholesterol efflux is unknown, association of ApoD on HDL is thought to be important to mediate the interaction of HDL_3 with LDL particles [51]. This may be a critical mechanism to drive the ability of HDL to prevent LDL oxidation. The higher levels of ApoD in men may therefore offer cardioprotective effects, by promoting HDL-LDL interactions. Further research on the potential causal role for ApoD in mediating CEC is necessary.

We finally wanted to investigate the relationship between the HDL proteome in men versus women to identify the degree of similarity in proteins governing HDL-CEC across both sexes. To our surprise there were remarkable differences in the proteins that correlated to HDL-CEC between the sexes, despite only moderate changes in HDL proteomic composition between men and women. Apolipoproteins on L-HDL-P (ApoA-I, ApoA-II, ApoC-I, ApoC-III, ApoC-IV, ApoD, ApoE, ApoF and ApoM) played a major role in driving total and ABCA1-independent efflux in women but to a much lesser extent in men (e.g. ApoA-II: r=0.574 in women and r=0.328 in men). Swertfeger *et al.,* also reported that apolipoproteins, ApoA-I, ApoA-II, and ApoC-I on fractions in the HDL range (total HDL; large and small fractions combined) strongly positively correlated with total CEC (r=0.993, r=0.951 and r=0.832 respectively) in non-obese, young men (*n*=10) [52]. In our study, we combined all sub-groups to investigate the relationship between the HDL proteome and HDL-CEC, resulting in potential loss of/ confounding effects due to the presence of obesity, T1DM and T2DM. In **Appendix 5F**, correlation analysis in the individual subgroups is reported and similarly to Swertfeger *et al.,* findings, in non-obese men, ApoA-II on L-HDL-P was strongly correlated to total efflux (r=0.693). We furthermore demonstrate striking disparity in the association between HDL associated Igs and HDL-CEC in women (positive associations) versus men (negative associations). The presence of Igs on HDL is a contentious issue with some believing them to be contaminants of HDL-containing FPLC fractions, as limited numbers are detectable on ultracentrifuged-derived HDL. However, we have included an extra purification step within our HDL-isolation preparation which involves precipitating phospholipid containing particles from FPLC fractions (i.e. HDL within the fractions) using LRA [53]. Even in the presence of this purification step, Igs are still present and their association with HDL has potentially detrimental consequences for HDL function in men, but not women. Similar to previous chapters the relationship between the HDL proteome and ABCA1-dependent CEC was weaker, but a significant correlation between ITIH4 on L-HDL-P and ABCA1-dependent CEC was evident in women (r=0.413) but not men (r=0.297).

This was consistent with our previous findings in Chapter 4 and validates ITIH4 as a potential biomarker of ABCA1-dependent CEC but in women more than men.

Similar to Igs, we found divergent effects in the association of complement proteins and coagulation proteins on S-HDL-P with total/ABCA1-independent CEC between men and women. In men, there were significant positive associations between coagulation factors and complement proteins on S-HDL-P and total/ABCA1-independent CEC (e.g. complement component c8 alpha chain ($r=0.441$ and $r=-0.184$ for men and women respectively in total CEC). Association of ApoC-I, ApoC-III and Apo-E on S-HDL-P in men, but not women, positively associated with ABCA1-dependent CEC, while a negative association to ApoD ($r=-0.405$) was evident. The apolipoproteins on L-HDL-P were more associated with total and ABCA1-independent CEC in women, while in men it was the apolipoproteins on S-HDL-P that were more associated with ABCA1-dependent CEC. Similar to L-HDL-P, the association between Igs on S-HDL-P in women, but not men was positively associated with total, and to a lesser extent ABCA1-independent CEC. Our findings demonstrate that despite only marginal differences within the HDL proteome between the sexes there are profound differences in the association of these proteins to functionality between the sexes. Our findings point to sex-specific differences in HDL-P size, composition and function, which may underpin enhanced CVD risk in women relative to men.

The study includes the following strengths: (1) inclusion of carefully age- and BMI-matched women and men of both, T1DM and T2DM diseases as well as respective age-, and BMI-matched controls for CEC and HDL-size analysis; (2) analysis of total, ABCA1-dependent and ABCA1-independent CEC and corresponding HDL-P size and concentration via NMR analysis; (4) profiling of both the large and small HDL proteome and (5) detailed characteristics of participants including smoking status and medications. An important limitation of this study, like the last chapter, is the inability to make causal inference. This study was cross-sectional; therefore, it was not designed to determine the longitudinal effect of either sex on HDL-P function and/or composition and subsequent incidence or progression of CVD. Another limitation was the correlation between HDL-proteome and HDL-CEC with all sub-groups combined due to limitation in the number of participants within the sub-groups – expanding these sub-groups in the future will be important to delineate sex-specific differences in proteins governing HDL-CEC in people with and without T1DM or T2DM. Unfortunately, oral contraceptive use is not known for all the women in this study. In general, oral contraceptives are associated with increases in HDL-C [54-56], although this may differ depending on the oestrogen and progestogen content [57]. Future studies should document women's contraceptive use to gain a greater understanding of how it might potential impact HDL-CEC.

Another potential confounder within our study is that the age range of the women (34-60 years) combines women both pre- and post-menopause (4/20 women across all groups were 45 years or older). Premenopausal women without diabetes are relatively protected from CVD compared to men [58]. Endogenous oestrogens are the proposed mechanism that reduces CVD risk [59], as women post-menopause have a 2.6 fold higher risk of CVD events compared to age-matched pre-menopausal women [60]. In addition, a meta-analysis showed that early-onset menopause was associated with an 1.5 elevated relative risk of CHD and 1.19 elevated relative risk for all-cause mortality, highlighting that early loss of oestrogens are harmful for cardiovascular health [61]. Indeed, post-menopause the risk of CVD in women approaches that of men, with older men having approximately a 1.5-2 times higher rates of CVD compared to women [1]. By contrast, young men have approximately 4 times higher rates of CVD compared to young women [1]. Badeau *et al.,* investigated the capacity of HDL derived from men and also women (mean BMI across groups 27.7-28.2) with high or low serum 17β oestradiol, the most bioactive member of oestrogen family, representing pre- and post-menopausal women respectively [62]. They found no significant differences in the efflux promoting abilities of HDL between the three groups, highlighting that oestradiol status or sex does not support a modifying role in the initial step of RCT in healthy men and women. However, the impact of changes in HDL-P and HDL-CEC over the menopausal transition on CVD risk later in life is not clear with much of our understanding of how the menopausal transition may impact HDL metrics coming from cross-sectional studies [63]. Future longitudinal studies are needed to evaluate the metrics of HDL so that the complex association of menopause, HDL metrics and CVD risk can be characterized.

In conclusion, our findings support the hypothesis that there are profound differences in HDL biology between the sexes. We demonstrate a reduction in HDL-CEC in women, but not men, with T2DM relative to non-obese controls; similarly, increased HDL-CEC evident in men with T1DM relative to their BMI-matched controls, was not observed in women with T1DM. These findings highlight a disadvantage in women with both T1DM and T2DM. We also demonstrated that the HDL proteome is moderately modulated between the sexes, but the proteins that govern HDL-CEC are remarkably different according to sex. Association of apolipoproteins and Igs on HDL-P in women positively correlates with HDL-CEC while association of Igs negatively correlates with HDL-CEC in men. By contrast, association of complement and coagulation proteins on S-HDL-P positively correlate with HDL-CEC in men but not women. Overall, our findings highlight the importance of studying the sexes separately and highlight that different protein biomarkers of HDL-CEC will likely be required for optimal stratified management of CVD risk across the sexes.

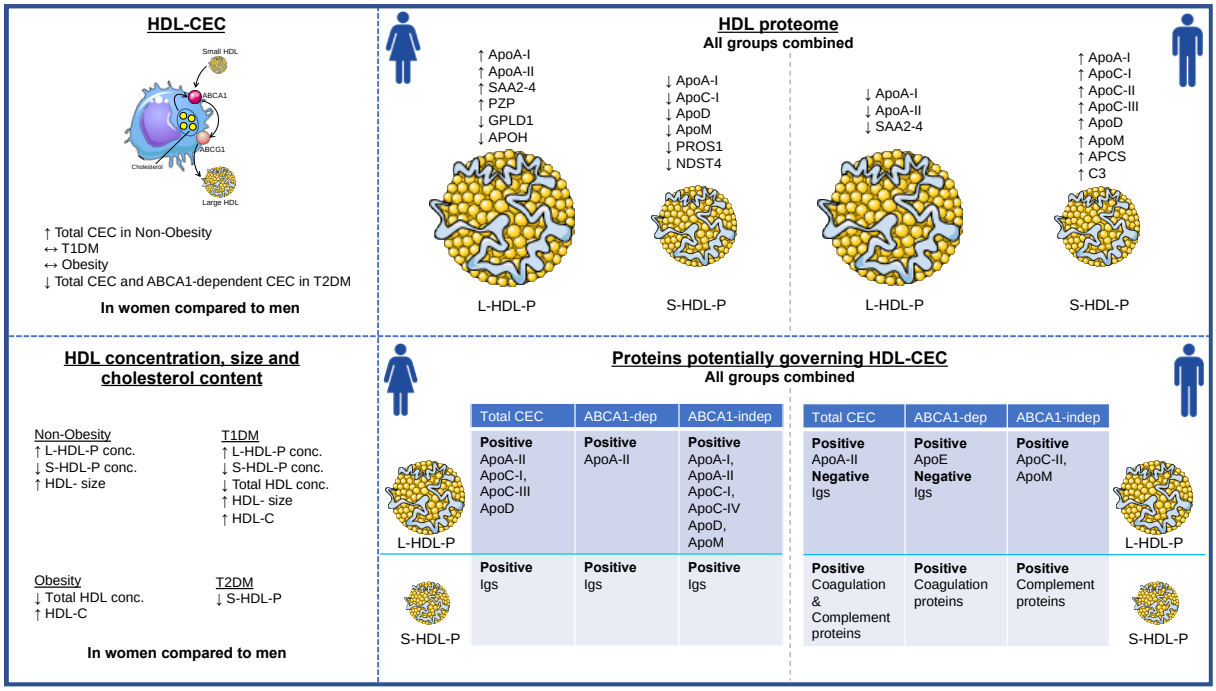

Figure 5.8 *Overall findings of chapter/Graphical abstract.*

5.5 References

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Appendix 5A – Medication Tables

Table 5.9 *Medication use in women and men across the groups.*

	Non-OB (*n*=20) %	Non-OB (*n*=20) %	T1DM (*n*=20) %	T1DM (*n*=20) %	OB (*n*=20) %	OB (*n*=20) %	T2DM (*n*=20) %	T2DM (*n*=20) %
	Women	Men	Women	Men	Women	Men	Women	Men
Medication use overall	0	0	30	50	5	0	70	90
Medication								
ACE-1/ARB (anti-hypertensive)	0	0	30	30	0	0	10	25
Gliclazide (insulin-sensitising)	0	0	0	0	0	0	15	20
DPP4 (insulin-sensitising)	0	0	0	0	0	0	10	10
Metformin (insulin-sensitising)	0	0	0	0	0	0	60	90
GLP-1	0	0	0	0	0	0	15	25
Statin	0	0	20	40	5▪	0	5	25

▪Person on statin oldest in group (Age 59)

Table 5.10 *Medication use in women and men across the groups in sub-cohort for proteomics analysis.*

	Non-OB (*n*=10) %	Non-OB (*n*=10) %	T1DM (*n*=10) %	T1DM (*n*=10) %	OB (*n*=10) %	OB (*n*=10) %	T2DM (*n*=10) %	T2DM (*n*=10) %
	Women	Men	Women	Men	Women	Men	Women	Men
Medication use overall	0	0	30	30	10	0	60	100
Medication								
ACE-1/ARB (anti-hypertensive)	0	0	30	10	0	0	10	30
Gliclazide (insulin-sensitising)	0	0	0	0	0	0	30	30
DPP4 (insulin-sensitising)	0	0	0	0	0	0	20	10
Metformin (insulin-sensitising)	0	0	0	0	0	0	40	100
GLP-1	0	0	0	0	0	0	10	40
Statin	0	0	30	20	10▪	0	10	30

▪Person on statin oldest in group (Age 59)

Appendix 5B – All proteins detected on L-HDL-P in women and men

216 proteins were detected on L-HDL-P in women and men and are shown below.

Table 5.11 *Proteins detected on L-HDL-P in women and men with and without T1DM or T2DM.*

Protein names	Majority protein IDs	Non-OB	T1DM	OB	T2DM	Non-OB	T1DM	OB	T2DM
	Uniprot	Mean LFQ Intensity Women				Mean LFQ Intensity Men			
A-kinase anchor protein 9	A0A2R8Y590	1.15E+08	1.21E+08	1.27E+08	1.07E+08	1.29E+08	1.13E+08	1.31E+08	7.84E+07
Actin, cytoplasmic 1	P60709	7.43E+07	8.63E+07	8.29E+07	7.87E+07	5.49E+07	8.80E+07	8.99E+07	7.57E+07
Adipocyte plasma membrane-associated protein	Q9HDC9	2.78E+08	5.04E+08	3.45E+08	3.86E+08	2.44E+08	5.59E+08	4.04E+08	4.20E+08
Afamin	P43652	4.72E+08	4.00E+08	5.21E+08	4.68E+08	3.94E+08	3.33E+08	5.29E+08	5.36E+08
Albumin	P02768	6.62E+10	7.51E+10	6.47E+10	6.09E+10	6.93E+10	6.60E+10	7.56E+10	6.88E+10
Alpha-1-acid glycoprotein 2	P19652	1.92E+08	2.31E+08	2.85E+08	2.21E+08	1.29E+08	1.82E+08	2.22E+08	2.61E+08
Alpha-1-antichymotrypsin	P01011	1.37E+09	1.53E+09	1.44E+09	1.29E+09	1.39E+09	1.36E+09	1.51E+09	1.40E+09
Alpha-1-antitrypsin	P01009	9.90E+09	8.24E+09	9.47E+09	5.37E+09	1.43E+10	6.44E+09	5.36E+09	4.75E+09
Alpha-1B-glycoprotein	P04217	5.38E+08	6.13E+08	6.41E+08	5.43E+08	6.07E+08	5.56E+08	5.53E+08	7.20E+08
Alpha-2-antiplasmin	P08697	6.52E+08	6.96E+08	5.73E+08	5.77E+08	6.68E+08	5.98E+08	6.27E+08	7.02E+08
Alpha-2-HS-glycoprotein	P02765	8.64E+08	8.72E+08	8.40E+08	7.22E+08	7.26E+08	7.91E+08	8.79E+08	9.64E+08
Alpha-2-macroglobulin	P01023	7.87E+09	8.09E+09	6.51E+09	6.68E+09	7.38E+09	8.59E+09	7.39E+09	8.31E+09
Angiotensinogen	A0A7P0T9S6	2.55E+08	2.16E+08	2.75E+08	2.37E+08	2.83E+08	2.31E+08	1.91E+08	2.10E+08
Antithrombin-III	P01008	8.65E+08	5.84E+08	8.54E+08	7.36E+08	1.12E+09	6.16E+08	7.52E+08	6.58E+08
Apolipoprotein A-I	P02647	1.86E+11	2.08E+11	1.22E+11	1.01E+11	1.26E+11	1.37E+11	8.65E+10	7.20E+10
Apolipoprotein A-II	P02652	1.72E+10	2.43E+10	1.60E+10	1.25E+10	1.41E+10	1.49E+10	1.28E+10	1.23E+10
Apolipoprotein A-IV	P06727	2.50E+10	3.33E+10	1.79E+10	4.16E+10	2.87E+10	2.53E+10	3.04E+10	7.51E+10
Apolipoprotein B-100	P04114	8.62E+08	8.41E+08	9.63E+08	8.65E+08	8.44E+08	7.93E+08	1.00E+09	1.06E+09
Apolipoprotein C-I	K7ERI9	1.49E+09	2.12E+09	1.63E+09	1.23E+09	1.36E+09	1.45E+09	1.88E+09	1.63E+09
Apolipoprotein C-II	K7ER74	1.80E+09	1.91E+09	2.31E+09	1.72E+09	1.54E+09	2.09E+09	1.74E+09	2.15E+09
Apolipoprotein C-III	P02656	3.23E+09	5.07E+09	3.46E+09	4.03E+09	3.54E+09	4.53E+09	3.09E+09	3.15E+09
Apolipoprotein C-IV	P55056	2.39E+08	2.14E+08	1.98E+08	2.27E+08	2.18E+08	2.31E+08	1.72E+08	1.68E+08

Apolipoprotein D	P05090	9.10E+09	1.13E+10	6.32E+09	5.19E+09	9.36E+09	9.81E+09	5.55E+09	5.01E+09
Apolipoprotein E	P02649	1.21E+10	1.34E+10	1.38E+10	1.02E+10	1.04E+10	1.24E+10	1.24E+10	2.03E+10
Apolipoprotein F	Q13790	4.14E+08	5.34E+08	2.79E+08	2.71E+08	4.02E+08	6.67E+08	2.88E+08	2.74E+08
Apolipoprotein L1	O14791	1.60E+09	1.59E+09	3.01E+09	2.88E+09	1.71E+09	1.32E+09	2.50E+09	3.26E+09
Apolipoprotein M	O95445	1.00E+10	1.17E+10	6.97E+09	7.90E+09	7.56E+09	1.08E+10	8.56E+09	6.27E+09
Attractin	O75882	1.17E+09	1.61E+09	1.66E+09	1.47E+09	1.68E+09	1.86E+09	1.62E+09	2.32E+09
Basement membrane-specific heparan sulfate proteoglycan core protein	P98160	2.26E+07	2.57E+07	2.74E+07	2.76E+07	2.57E+07	2.86E+07	3.40E+07	3.00E+07
Beta-2-glycoprotein 1	P02749	2.74E+08	4.27E+08	3.46E+08	2.27E+08	3.72E+08	4.11E+08	3.97E+08	3.98E+08
Beta-Ala-His dipeptidase	Q96KN2	7.45E+08	6.84E+08	7.38E+08	8.34E+08	5.97E+08	5.95E+08	5.08E+08	7.21E+08
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	Q9H3R1	4.92E+08	9.58E+08	6.97E+08	6.69E+08	1.07E+09	8.11E+08	7.41E+08	5.26E+08
C3/C5 convertase	B4E1Z4	1.73E+09	2.12E+09	2.23E+09	1.69E+09	1.45E+09	1.63E+09	1.88E+09	2.31E+09
C4b-binding protein alpha chain	P04003	5.05E+08	7.19E+08	5.37E+08	5.76E+08	4.43E+08	5.53E+08	5.89E+08	5.60E+08
Cadherin-13	P55290	1.05E+08	9.83E+07	1.12E+08	1.00E+08	1.33E+08	1.33E+08	1.38E+08	1.09E+08
Cadherin-5	P33151	6.07E+08	6.40E+08	6.62E+08	5.49E+08	7.23E+08	6.75E+08	5.44E+08	6.26E+08
Carboxypeptidase N catalytic chain	P15169	5.74E+08	6.21E+08	8.26E+08	7.49E+08	5.72E+08	5.10E+08	8.28E+08	6.96E+08
Carboxypeptidase N subunit 2	P22792	8.38E+08	7.75E+08	9.53E+08	1.12E+09	1.28E+09	1.01E+09	1.40E+09	9.10E+08
Cartilage acidic protein 1	Q9NQ79	2.20E+08	2.26E+08	2.39E+08	1.97E+08	2.50E+08	1.68E+08	1.65E+08	2.00E+08
Cartilage oligomeric matrix protein	G3XAP6	4.64E+08	4.82E+08	5.81E+08	5.44E+08	5.83E+08	6.11E+08	7.46E+08	5.96E+08
Cathelicidin antimicrobial peptide	P49913	5.53E+07	6.09E+07	4.46E+07	4.69E+07	4.33E+07	3.76E+07	5.02E+07	5.90E+07
CD5 antigen-like	O43866	1.61E+08	1.40E+08	1.02E+08	1.11E+08	1.11E+08	1.08E+08	9.22E+07	1.18E+08
Cell surface glycoprotein MUC18	P43121	8.22E+07	9.89E+07	8.84E+07	8.48E+07	9.51E+07	1.03E+08	9.16E+07	1.01E+08
Ceruloplasmin	P00450	7.94E+09	1.12E+10	8.80E+09	8.20E+09	8.85E+09	7.84E+09	7.17E+09	6.61E+09
Clusterin	P10909	9.69E+09	8.53E+09	1.22E+10	1.20E+10	1.09E+10	1.12E+10	1.22E+10	1.24E+10
Coagulation factor V	P12259	7.88E+07	7.70E+07	8.93E+07	9.67E+07	5.43E+07	8.95E+07	6.76E+07	7.11E+07
Coagulation factor X	P00742	1.09E+08	1.03E+08	1.18E+08	1.36E+08	9.51E+07	1.02E+08	9.82E+07	1.38E+08
Coagulation factor XII	P00748	6.84E+07	1.12E+08	8.82E+07	9.78E+07	1.82E+08	5.19E+07	3.98E+07	5.04E+07
Coagulation factor XIII B chain	P05160	3.04E+09	2.91E+09	2.80E+09	3.15E+09	2.62E+09	2.88E+09	3.00E+09	2.50E+09
Complement C1q subcomponent subunit B (Fragment)	A0A0A0MSV6	1.44E+08	9.27E+07	1.54E+08	1.50E+08	1.05E+08	1.00E+08	1.09E+08	1.39E+08
Complement C1q subcomponent subunit C	P02747	1.70E+08	1.42E+08	1.92E+08	1.99E+08	1.29E+08	1.55E+08	1.52E+08	1.53E+08

Complement C1r subcomponent-like protein	Q9NZP8	8.01E+07	1.11E+08	1.14E+08	9.35E+07	7.37E+07	7.68E+07	1.21E+08	9.64E+07
Complement C1s subcomponent	P09871	2.05E+10	1.83E+10	1.79E+10	1.94E+10	1.93E+10	2.24E+10	2.01E+10	1.96E+10
Complement C3	P01024	2.27E+11	1.85E+11	2.79E+11	2.51E+11	2.04E+11	1.98E+11	2.18E+11	2.19E+11
Complement C4-A	P0C0L4	1.53E+09	1.80E+09	1.74E+09	1.96E+09	1.86E+09	1.97E+09	1.75E+09	2.14E+09
Complement C4-B	P0C0L5	1.88E+11	1.67E+11	2.03E+11	2.32E+11	1.86E+11	1.93E+11	2.06E+11	2.20E+11
Complement C5	P01031	2.30E+10	2.18E+10	2.72E+10	2.81E+10	1.89E+10	2.21E+10	2.50E+10	2.70E+10
Complement component C6	P13671	1.13E+10	9.75E+09	1.13E+10	1.11E+10	8.97E+09	9.90E+09	1.07E+10	9.74E+09
Complement component C7	P10643	4.02E+09	4.07E+09	3.87E+09	3.92E+09	3.28E+09	4.10E+09	3.11E+09	4.04E+09
Complement component C8 alpha chain	P07357	6.11E+07	4.68E+07	6.26E+07	4.16E+07	4.83E+07	5.48E+07	5.53E+07	7.16E+07
Complement component C8 beta chain	P07358	6.68E+06	2.22E+07	1.18E+07	8.10E+06	7.23E+06	1.45E+07	1.18E+07	1.23E+07
Complement component C8 gamma chain	P07360	1.12E+07	1.81E+07	1.61E+07	1.84E+07	1.12E+07	1.88E+07	1.85E+07	1.69E+07
Complement component C9	P02748	4.87E+07	6.81E+07	8.24E+07	6.95E+07	4.34E+07	4.26E+07	5.03E+07	6.95E+07
Complement factor H	P08603	6.42E+09	6.16E+09	7.79E+09	7.41E+09	6.51E+09	6.11E+09	7.10E+09	7.42E+09
Complement factor H-related protein 1	Q03591	1.69E+08	2.15E+08	2.12E+08	1.50E+08	1.44E+08	1.64E+08	1.79E+08	2.43E+08
Complement factor H-related protein 2	P36980	5.40E+07	7.03E+07	5.29E+07	3.57E+07	2.29E+07	5.00E+07	2.79E+07	4.96E+07
Complement factor I	G3XAM2	2.37E+08	6.47E+08	2.88E+08	2.68E+08	2.00E+08	2.49E+08	3.14E+08	3.05E+08
Complement subcomponent C1r	A0A3B3ISR2	1.61E+09	1.37E+09	1.74E+09	1.77E+09	1.77E+09	1.74E+09	1.79E+09	1.61E+09
Endoplasmic reticulum chaperone BiP	P11021	9.81E+07	1.08E+08	9.60E+07	1.09E+08	6.82E+07	8.79E+07	1.07E+08	1.07E+08
Endoplasmin	A0A7P0T917	5.16E+07	4.54E+07	6.25E+07	4.19E+07	4.14E+07	6.17E+07	4.43E+07	6.68E+07
Extracellular matrix protein 1	Q16610	3.61E+08	2.98E+08	2.97E+08	3.43E+08	2.41E+08	2.98E+08	3.89E+08	3.03E+08
Extracellular superoxide dismutase [Cu-Zn]	P08294	2.65E+07	2.53E+07	4.19E+07	3.12E+08	2.42E+07	3.89E+07	2.74E+07	2.21E+07
Fibrinogen alpha chain	P02671	6.66E+07	6.44E+07	8.22E+07	6.46E+07	4.91E+07	6.64E+07	9.91E+07	9.04E+07
Fibronectin	P02751	1.43E+09	2.09E+09	1.57E+09	1.42E+09	1.19E+09	1.28E+09	1.81E+09	1.48E+09
Fibulin-1	P23142	3.83E+09	3.73E+09	3.36E+09	3.45E+09	3.47E+09	3.62E+09	3.81E+09	3.18E+09
Fibulin-1	B1AHL2	4.86E+07	5.49E+07	4.83E+07	5.32E+07	5.80E+07	5.61E+07	6.36E+07	5.38E+07
Ficolin-3	O75636	4.49E+07	3.23E+07	4.23E+07	4.77E+07	5.10E+07	3.25E+07	5.73E+07	5.91E+07
GDH/6PGL endoplasmic bifunctional protein	O95479	4.58E+07	3.96E+07	6.81E+07	6.44E+07	5.14E+07	1.40E+08	4.23E+07	7.01E+07
Gelsolin	P06396	5.11E+08	5.31E+08	5.23E+08	4.26E+08	5.13E+08	5.38E+08	6.88E+08	5.30E+08
Haptoglobin	P00738	1.69E+11	1.05E+11	7.16E+10	1.27E+11	1.17E+11	1.93E+11	1.26E+11	8.46E+10

Haptoglobin-related protein	P00739	6.56E+08	4.59E+08	2.84E+08	3.01E+08	5.06E+08	7.59E+08	5.22E+08	4.13E+08
Hemoglobin subunit alpha	P69905	2.95E+09	4.52E+09	2.49E+09	3.27E+09	4.56E+09	7.61E+09	2.91E+09	4.35E+09
Hemoglobin subunit beta	P68871	3.20E+09	6.18E+09	3.41E+09	6.37E+09	8.30E+09	8.46E+09	3.17E+09	9.83E+09
Hemopexin	P02790	1.42E+09	1.51E+09	1.65E+09	1.52E+09	1.44E+09	1.42E+09	1.67E+09	1.86E+09
Heparin cofactor 2	P05546	5.47E+08	5.97E+08	6.52E+08	5.79E+08	4.75E+08	5.24E+08	6.34E+08	7.51E+08
Hepatocyte growth factor activator	Q04756	2.34E+09	2.17E+09	2.37E+09	2.22E+09	1.77E+09	2.18E+09	2.43E+09	2.79E+09
Histidine-rich glycoprotein	P04196	8.15E+08	9.28E+08	9.15E+08	9.16E+08	1.08E+09	8.30E+08	9.14E+08	9.94E+08
Histone H2A	A0A0U1RRH7	1.31E+08	1.59E+08	1.43E+08	1.50E+08	1.12E+08	1.59E+08	1.79E+08	1.38E+08
Hyaluronan-binding protein 2	Q14520	3.73E+07	4.17E+07	4.56E+07	3.63E+07	4.62E+07	3.75E+07	4.46E+07	4.27E+07
Hypoxia up-regulated protein 1	A0A087X054	3.24E+07	2.68E+07	2.38E+07	2.43E+07	2.62E+07	2.73E+07	2.38E+07	2.67E+07
Ig-like domain-containing protein (Fragment)	A0A0G2JRQ6	3.59E+08	3.19E+08	3.42E+08	3.41E+08	3.49E+08	4.23E+08	3.62E+08	4.23E+08
Ig-like domain-containing protein (Fragment)	A0A0J9YY99	5.38E+09	6.36E+09	4.95E+09	6.85E+09	6.30E+09	6.48E+09	5.62E+09	3.60E+09
Immunoglobulin heavy constant alpha 1	P01876	1.05E+11	9.88E+10	8.45E+10	1.03E+11	1.44E+11	1.20E+11	1.06E+11	8.53E+10
Immunoglobulin heavy constant alpha 2 (Fragment)	A0A0G2JMB2	4.04E+09	6.42E+09	4.54E+09	3.59E+09	5.28E+09	6.12E+09	4.68E+09	5.43E+09
Immunoglobulin heavy constant delta	P01880	1.59E+09	7.19E+08	2.54E+09	2.50E+09	2.46E+09	1.13E+09	5.41E+08	1.33E+09
Immunoglobulin heavy constant gamma 1	P01857	5.36E+10	4.94E+10	4.79E+10	5.00E+10	6.36E+10	6.27E+10	5.28E+10	4.36E+10
Immunoglobulin heavy constant gamma 2	P01859	2.75E+10	2.88E+10	2.79E+10	2.50E+10	2.57E+10	2.52E+10	2.31E+10	1.97E+10
Immunoglobulin heavy constant gamma 3	P01860	4.54E+10	5.03E+10	4.53E+10	4.60E+10	5.00E+10	4.65E+10	4.26E+10	3.27E+10
Immunoglobulin heavy constant gamma 4	P01861	6.19E+09	6.35E+09	4.43E+09	3.83E+09	8.58E+09	6.80E+09	9.84E+09	8.40E+09
Immunoglobulin heavy constant mu	P01871	3.28E+09	4.24E+09	3.13E+09	2.93E+09	2.64E+09	2.53E+09	2.62E+09	3.33E+09
Immunoglobulin heavy variable 1-18	A0A0C4DH31	9.30E+08	8.55E+08	9.13E+08	1.07E+09	1.02E+09	1.18E+09	9.54E+08	8.69E+08
Immunoglobulin heavy variable 1-2	P23083	3.14E+09	4.20E+09	2.82E+09	3.01E+09	2.78E+09	2.34E+09	2.70E+09	2.16E+09
Immunoglobulin heavy variable 1-24	A0A0C4DH33	6.96E+07	1.13E+08	4.00E+07	1.04E+08	1.24E+08	1.54E+08	9.03E+07	8.10E+07
Immunoglobulin heavy variable 1-45	A0A0A0MS14	1.69E+08	9.80E+07	1.60E+08	1.43E+08	1.43E+08	1.08E+08	1.06E+08	1.65E+08
Immunoglobulin heavy variable 1-46	P01743	8.86E+07	7.69E+07	1.03E+08	1.20E+08	1.93E+08	9.66E+07	8.81E+07	6.64E+07
Immunoglobulin heavy variable 1-69	P01742	2.16E+09	2.38E+09	2.64E+09	2.77E+09	1.93E+09	2.53E+09	2.38E+09	2.08E+09
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	A0A075B7D0	7.69E+08	1.26E+09	1.77E+09	9.19E+08	1.15E+09	1.26E+09	9.11E+08	9.69E+08
Immunoglobulin heavy variable 2-26	A0A0B4J1V2	8.57E+07	1.29E+08	1.23E+08	1.30E+08	8.89E+07	1.44E+08	7.70E+07	6.48E+07
Immunoglobulin heavy variable 2-70D	A0A0C4DH43	8.05E+08	6.42E+08	1.29E+09	1.08E+09	7.29E+08	8.08E+08	1.90E+09	6.42E+08

Immunoglobulin heavy variable 3-13	P01766	3.15E+08	2.62E+08	2.92E+08	3.68E+08	3.55E+08	3.13E+08	2.61E+08	3.04E+08
Immunoglobulin heavy variable 3-15	A0A0B4J1V0	1.06E+09	9.99E+08	1.11E+09	1.05E+09	5.79E+08	7.25E+08	8.69E+08	6.11E+08
Immunoglobulin heavy variable 3-43	A0A0B4J1X8	8.52E+07	1.73E+08	1.34E+08	2.54E+08	1.49E+08	2.29E+08	1.41E+08	1.40E+08
Immunoglobulin heavy variable 3-49	A0A0A0MS15	5.19E+08	7.62E+08	7.35E+08	6.40E+08	7.99E+08	6.27E+08	4.88E+08	4.26E+08
Immunoglobulin heavy variable 3-64D	A0A0J9YX35	2.98E+08	3.82E+08	2.67E+08	5.44E+08	3.79E+08	3.00E+08	3.54E+08	1.54E+08
Immunoglobulin heavy variable 3-7	P01780	7.37E+09	7.93E+09	8.47E+09	7.94E+09	7.12E+09	7.00E+09	6.40E+09	5.44E+09
Immunoglobulin heavy variable 3-72	A0A4W8ZXM2	7.55E+09	8.82E+09	9.33E+09	8.56E+09	7.81E+09	6.65E+09	8.09E+09	6.92E+09
Immunoglobulin heavy variable 3-73	A0A0B4J1V6	3.91E+08	5.24E+08	5.09E+08	3.72E+08	3.39E+08	3.54E+08	4.69E+08	3.44E+08
Immunoglobulin heavy variable 3-74	A0A0B4J1X5	9.90E+08	1.26E+09	1.34E+09	1.35E+09	6.86E+08	7.40E+08	9.56E+08	8.30E+08
Immunoglobulin heavy variable 3-9	P01782	1.01E+09	6.50E+08	9.06E+08	8.04E+08	8.77E+08	1.24E+09	6.07E+08	6.59E+08
Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Fragment)	A0A075B7B8	1.51E+09	2.41E+09	2.29E+09	2.23E+09	2.08E+09	1.70E+09	2.53E+09	2.51E+09
Immunoglobulin heavy variable 4-28	A0A0C4DH34	1.19E+09	1.29E+09	1.21E+09	1.82E+09	1.19E+09	1.38E+09	1.01E+09	9.85E+08
Immunoglobulin heavy variable 4-38-2	P0DP08	1.22E+09	1.46E+09	1.44E+09	1.62E+09	1.06E+09	1.27E+09	1.08E+09	1.19E+09
Immunoglobulin heavy variable 4-4	A0A075B6R2	6.93E+08	1.03E+09	9.17E+08	6.83E+08	1.14E+09	7.73E+08	7.73E+08	6.15E+08
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	A0A075B7B6	2.69E+08	2.60E+08	3.81E+08	4.06E+08	2.50E+08	2.85E+08	3.22E+08	2.38E+08
Immunoglobulin heavy variable 5-51	A0A0C4DH38	2.16E+09	1.93E+09	1.68E+09	2.11E+09	2.14E+09	2.37E+09	1.86E+09	1.35E+09
Immunoglobulin heavy variable 6-1	A0A0B4J1U7	1.71E+09	1.78E+09	3.73E+09	3.22E+09	1.29E+09	1.88E+09	2.62E+09	3.19E+09
Immunoglobulin J chain	P01591	3.94E+08	4.47E+08	3.70E+08	3.30E+08	2.17E+08	3.62E+08	3.65E+08	3.45E+08
Immunoglobulin kappa constant	P01834	4.13E+10	3.88E+10	3.26E+10	3.54E+10	4.86E+10	4.92E+10	3.83E+10	3.38E+10
Immunoglobulin kappa variable 1-17	P01599	1.40E+08	1.68E+08	1.90E+08	1.66E+08	1.83E+08	1.79E+08	1.75E+08	1.12E+08
Immunoglobulin kappa variable 1-33	A0A2Q2TTZ9	2.80E+09	3.36E+09	3.54E+09	3.25E+09	4.48E+09	3.29E+09	2.82E+09	2.51E+09
Immunoglobulin kappa variable 1-6	A0A0C4DH72	8.45E+07	9.17E+07	7.09E+07	8.63E+07	8.19E+07	9.10E+07	7.75E+07	8.82E+07
Immunoglobulin kappa variable 1-8	A0A0C4DH67	1.13E+08	1.22E+08	1.94E+08	1.51E+08	1.56E+08	1.32E+08	1.16E+08	1.32E+08
Immunoglobulin kappa variable 2-24	A0A0C4DH68	8.79E+08	7.84E+08	8.43E+08	1.28E+09	6.78E+08	9.58E+08	6.67E+08	7.34E+08
Immunoglobulin kappa variable 2-30	P06310	6.79E+08	9.18E+08	1.04E+09	9.34E+08	6.01E+08	7.91E+08	6.68E+08	6.18E+08
Immunoglobulin kappa variable 2-40	A0A087X0Q4	7.52E+09	9.06E+09	6.95E+09	8.06E+09	1.02E+10	9.41E+09	1.12E+10	8.17E+09
Immunoglobulin kappa variable 2D-28	A0A5H1ZRS2	5.52E+08	4.75E+08	4.08E+08	3.00E+08	6.23E+08	5.40E+08	5.28E+08	4.36E+08
Immunoglobulin kappa variable 2D-29	A0A5H1ZRS9	3.61E+08	3.67E+08	5.82E+08	2.62E+08	3.92E+08	3.36E+08	5.21E+08	4.22E+08
Immunoglobulin kappa variable 3-11	P04433	5.21E+09	5.41E+09	7.44E+09	5.07E+09	5.85E+09	4.80E+09	7.67E+09	6.17E+09

Immunoglobulin kappa variable 3-20	P01619	2.50E+09	2.65E+09	2.31E+09	1.80E+09	2.39E+09	2.39E+09	2.95E+09	3.03E+09
Immunoglobulin kappa variable 4-1	P06312	1.17E+09	1.21E+09	1.55E+09	1.59E+09	1.52E+09	1.36E+09	1.47E+09	1.36E+09
Immunoglobulin kappa variable 6-21	A0A0C4DH24	9.26E+07	1.11E+08	9.68E+07	1.41E+08	1.99E+08	1.33E+08	1.07E+08	9.59E+07
Immunoglobulin lambda constant 2	P0DOY2	4.53E+07	3.94E+07	2.75E+07	9.79E+07	7.76E+07	2.20E+07	2.36E+08	3.74E+07
Immunoglobulin lambda constant 3	P0DOY3	3.82E+10	3.70E+10	3.23E+10	3.58E+10	4.61E+10	3.83E+10	3.58E+10	3.15E+10
Immunoglobulin lambda constant 7 (Fragment)	A0A5H1ZRQ7	1.88E+08	1.77E+08	1.34E+08	1.63E+08	1.89E+08	1.55E+08	3.86E+08	1.83E+08
Immunoglobulin lambda variable 1-47	P01700	2.18E+09	1.76E+09	2.00E+09	2.01E+09	2.24E+09	1.64E+09	1.59E+09	1.33E+09
Immunoglobulin lambda variable 1-51	P01701	1.37E+08	1.69E+08	1.96E+08	2.04E+08	1.61E+08	1.71E+08	1.37E+08	1.61E+08
Immunoglobulin lambda variable 2-11	P01706	1.79E+08	1.93E+08	1.58E+08	1.44E+08	1.48E+08	1.68E+08	1.45E+08	1.71E+08
Immunoglobulin lambda variable 3-10	A0A075B6K4	1.78E+09	2.17E+09	2.34E+09	3.03E+09	2.87E+09	2.03E+09	2.66E+09	1.74E+09
Immunoglobulin lambda variable 3-16	A0A075B6K0	7.78E+08	9.56E+08	9.92E+08	7.34E+08	6.75E+08	6.64E+08	6.86E+08	6.85E+08
Immunoglobulin lambda variable 3-19	P01714	7.39E+08	7.85E+08	7.71E+08	7.92E+08	6.47E+08	6.48E+08	8.39E+08	8.39E+08
Immunoglobulin lambda variable 3-21	P80748	2.09E+08	1.31E+08	1.58E+08	1.55E+08	2.48E+08	2.02E+08	2.59E+08	1.79E+08
Immunoglobulin lambda variable 3-9	A0A075B6K5	1.89E+09	1.85E+09	2.18E+09	1.94E+09	2.29E+09	1.71E+09	1.97E+09	1.72E+09
Immunoglobulin lambda variable 4-69	A0A075B6H9	1.38E+08	1.22E+08	1.16E+08	1.04E+08	1.15E+08	1.03E+08	1.31E+08	9.97E+07
Immunoglobulin lambda variable 5-45 (Fragment)	A0A0G2JSC0	5.49E+07	5.54E+07	3.49E+07	5.27E+07	5.19E+07	3.69E+07	5.79E+07	3.70E+07
Immunoglobulin lambda variable 7-46	A0A075B6I9	4.73E+08	5.49E+08	5.77E+08	4.95E+08	4.66E+08	4.86E+08	5.25E+08	6.14E+08
Immunoglobulin lambda variable 8-61	A0A075B6I0	4.35E+08	9.91E+08	4.95E+08	5.16E+08	2.98E+08	4.78E+08	3.36E+08	5.11E+08
Immunoglobulin lambda variable 9-49	A0A0B4J1Y8	8.37E+07	1.40E+08	1.11E+08	1.16E+08	2.08E+08	8.05E+07	7.46E+07	1.34E+08
Immunoglobulin lambda-like polypeptide 5	B9A064	5.05E+09	6.32E+09	6.17E+09	6.21E+09	6.41E+09	4.99E+09	6.78E+09	5.67E+09
Insulin-like growth factor-binding protein 3	A6XND1	5.28E+07	4.02E+07	4.78E+07	6.59E+07	3.65E+07	4.06E+07	3.39E+07	5.83E+07
Insulin-like growth factor-binding protein complex acid labile subunit	P35858	3.07E+08	2.56E+08	2.89E+08	3.10E+08	2.52E+08	1.89E+08	2.19E+08	2.14E+08
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	2.08E+10	2.33E+10	2.35E+10	2.05E+10	2.22E+10	2.03E+10	2.60E+10	2.27E+10
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	4.37E+10	4.40E+10	4.04E+10	3.97E+10	4.76E+10	3.94E+10	4.80E+10	4.27E+10
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033	7.59E+09	1.00E+10	7.28E+09	6.20E+09	6.41E+09	9.56E+09	6.83E+09	6.18E+09
Intercellular adhesion molecule 1	P05362	6.69E+07	6.76E+07	7.64E+07	6.83E+07	5.46E+07	9.61E+07	6.79E+07	7.40E+07
Interleukin-1 receptor accessory protein	Q9NPH3	5.72E+07	8.55E+07	3.28E+07	3.34E+07	3.00E+07	2.93E+07	3.09E+07	6.23E+07
ITIH4 protein	B7ZKJ8	4.10E+09	4.52E+09	4.17E+09	4.20E+09	3.05E+09	3.09E+09	3.91E+09	3.92E+09
Kallistatin	P29622	3.46E+07	3.70E+07	3.82E+07	3.12E+07	3.98E+07	3.86E+07	3.91E+07	3.86E+07

Keratin, type I cytoskeletal 10	P13645	3.18E+08	9.54E+08	2.53E+08	2.91E+08	1.61E+08	1.92E+08	2.15E+08	2.40E+08
Keratin, type I cytoskeletal 9	P35527	1.92E+08	1.27E+08	5.56E+07	1.34E+08	1.18E+08	9.20E+07	1.03E+08	2.34E+08
Keratin, type II cytoskeletal 1	P04264	5.29E+08	6.46E+08	3.27E+08	3.98E+08	2.98E+08	2.61E+08	2.63E+08	4.65E+08
Kininogen-1	P01042	1.31E+10	1.49E+10	1.32E+10	1.50E+10	1.40E+10	1.40E+10	1.45E+10	1.77E+10
L-selectin	P14151	5.85E+08	4.81E+08	4.24E+08	3.50E+08	5.34E+08	4.80E+08	4.00E+08	3.58E+08
Low affinity immunoglobulin gamma Fc region receptor III-A	P08637	2.43E+08	2.87E+08	2.55E+08	3.18E+08	2.47E+08	2.98E+08	3.56E+08	2.65E+08
Lumican	P51884	1.95E+09	2.18E+09	1.78E+09	2.00E+09	2.09E+09	2.51E+09	1.86E+09	1.59E+09
Lymphatic vessel endothelial hyaluronic acid receptor 1	Q9Y5Y7	5.40E+07	7.13E+07	7.25E+07	6.70E+07	5.68E+07	7.27E+07	6.66E+07	7.97E+07
Macrophage colony-stimulating factor 1 receptor	P07333	8.21E+07	9.33E+07	5.81E+07	8.72E+07	7.48E+07	8.92E+07	7.71E+07	8.52E+07
Mannan-binding lectin serine protease 1	P48740	5.26E+08	5.89E+08	5.96E+08	7.56E+08	5.69E+08	6.01E+08	6.09E+08	7.30E+08
Mannan-binding lectin serine protease 2	O00187	1.04E+08	1.15E+08	1.08E+08	1.38E+08	1.05E+08	1.30E+08	1.15E+08	1.24E+08
Mast/stem cell growth factor receptor Kit	P10721	1.26E+10	1.42E+10	1.38E+10	1.05E+10	1.36E+10	1.33E+10	1.28E+10	1.26E+10
Matrix metalloproteinase-9	P14780	7.33E+07	1.08E+08	5.98E+07	1.06E+08	8.57E+07	7.29E+07	9.68E+07	6.89E+07
N-acetylmuramoyl-L-alanine amidase	Q96PD5	4.77E+07	5.22E+07	4.79E+07	5.12E+07	5.19E+07	4.78E+07	4.54E+07	7.83E+07
Phosphatidylcholine-sterol acyltransferase	P04180	4.20E+08	4.61E+08	4.77E+08	4.74E+08	4.06E+08	4.69E+08	4.31E+08	5.11E+08
Phosphatidylinositol-glycan-specific phospholipase D	P80108	2.30E+09	2.52E+09	3.13E+09	3.90E+09	4.53E+09	3.89E+09	4.72E+09	5.92E+09
Phospholipid transfer protein	P55058	1.16E+08	9.73E+07	1.15E+08	1.10E+08	1.13E+08	1.02E+08	1.40E+08	1.02E+08
Plasma kallikrein	P03952	1.45E+09	1.53E+09	1.33E+09	1.69E+09	1.09E+09	1.27E+09	1.35E+09	3.01E+09
Plasma protease C1 inhibitor	A0A7I2V2D2	3.70E+10	3.64E+10	3.90E+10	3.42E+10	4.30E+10	4.99E+10	3.90E+10	4.92E+10
Plasminogen	P00747	2.31E+08	2.94E+08	2.79E+08	2.41E+08	2.34E+08	2.44E+08	2.51E+08	3.20E+08
Platelet factor 4	P02776	8.22E+07	9.38E+07	7.75E+07	7.94E+07	1.09E+08	1.08E+08	8.59E+07	9.39E+07
Plexin domain-containing protein 2	Q6UX71	8.85E+07	9.91E+07	1.06E+08	1.36E+08	9.20E+07	1.03E+08	1.30E+08	1.26E+08
Pregnancy zone protein	P20742	5.01E+08	1.46E+08	1.03E+09	7.28E+08	9.70E+07	5.10E+07	2.29E+08	1.48E+08
Prenylcysteine oxidase 1	Q9UHG3	4.60E+08	4.80E+08	4.04E+08	4.44E+08	3.71E+08	4.02E+08	3.77E+08	3.02E+08
Probable non-functional immunoglobulin heavy variable 3-38	A0A0C4DH36	2.57E+08	2.93E+08	2.96E+08	3.56E+08	3.34E+08	2.39E+08	1.86E+08	2.46E+08
Properdin	P27918	3.17E+08	2.65E+08	3.75E+08	4.17E+08	3.19E+08	2.84E+08	5.72E+08	4.23E+08
Protein AMBP	P02760	1.05E+10	1.28E+10	1.36E+10	1.20E+10	1.43E+10	1.24E+10	1.31E+10	1.31E+10
Protein Z-dependent protease inhibitor	Q9UK55	3.51E+07	5.19E+07	3.87E+07	3.85E+07	3.80E+07	3.74E+07	4.78E+07	3.70E+07
Prothrombin	P00734	6.04E+09	3.77E+09	5.59E+09	5.98E+09	5.67E+09	4.97E+09	5.94E+09	5.25E+09

SAA2-SAA4	A0A096LPE2	6.07E+09	7.88E+09	4.70E+09	4.62E+09	4.03E+09	5.61E+09	3.96E+09	3.85E+09
Scavenger receptor cysteine-rich type 1 protein M130	F5GZZ9	9.08E+07	9.49E+07	1.57E+08	1.38E+08	1.19E+08	1.29E+08	2.13E+08	1.59E+08
Selenoprotein P	P49908	6.40E+08	7.96E+08	7.60E+08	8.41E+08	6.84E+08	6.70E+08	7.31E+08	1.06E+09
Serotransferrin	P02787	4.79E+09	5.80E+09	5.05E+09	4.95E+09	5.14E+09	5.02E+09	5.69E+09	5.72E+09
Serum amyloid P-component	P02743	2.84E+08	7.53E+07	1.22E+08	1.79E+08	3.63E+07	6.27E+07	8.55E+07	1.21E+08
Serum paraoxonase/arylesterase 1	P27169	1.21E+10	1.06E+10	8.02E+09	8.59E+09	1.03E+10	1.01E+10	7.39E+09	6.99E+09
Serum paraoxonase/lactonase 3	Q15166	3.18E+08	2.49E+08	2.07E+08	1.69E+08	2.63E+08	3.28E+08	2.01E+08	1.40E+08
Sulfhydryl oxidase 1	O00391	1.18E+08	1.29E+08	1.51E+08	1.50E+08	1.19E+08	1.45E+08	1.23E+08	1.68E+08
Tetranectin	E9PHK0	1.68E+07	1.17E+07	1.03E+07	9.15E+06	9.86E+06	1.37E+07	1.58E+07	1.47E+07
Transferrin receptor protein 1	P02786	8.07E+08	9.60E+08	6.75E+08	1.08E+09	6.93E+08	9.29E+08	8.04E+08	9.94E+08
Transforming growth factor-beta-induced protein ig-h3	Q15582	5.24E+07	5.54E+07	4.47E+07	5.72E+07	4.96E+07	6.01E+07	4.30E+07	4.53E+07
Transthyretin	A0A087WT59	1.58E+08	1.30E+08	1.38E+08	1.26E+08	1.54E+08	1.32E+08	1.87E+08	1.84E+08
Uncharacterized protein C16orf46	Q6P387	1.55E+09	1.63E+09	1.17E+09	1.25E+09	9.97E+08	1.12E+09	1.34E+09	9.60E+08
Vasorin	Q6EMK4	1.69E+08	1.41E+08	1.65E+08	1.63E+08	2.29E+08	2.16E+08	2.00E+08	2.14E+08
Vitamin D-binding protein	P02774	1.90E+08	1.83E+08	2.47E+08	2.56E+08	1.91E+08	2.01E+08	2.87E+08	2.55E+08
Vitamin K-dependent protein C	P04070	1.03E+07	1.15E+07	8.86E+06	7.92E+06	8.88E+06	1.04E+07	8.99E+06	1.10E+07
Vitamin K-dependent protein S	A0A3B3ISJ1	7.27E+08	7.91E+08	8.33E+08	8.75E+08	7.78E+08	7.96E+08	8.26E+08	9.53E+08
Vitamin K-dependent protein Z	P22891	9.22E+07	6.67E+07	1.17E+08	9.98E+07	8.40E+07	1.03E+08	1.13E+08	1.68E+08
Vitronectin	P04004	5.32E+09	4.80E+09	6.02E+09	6.22E+09	5.72E+09	4.92E+09	5.77E+09	6.43E+09
von Willebrand factor	P04275	1.98E+08	1.31E+08	1.82E+08	2.19E+08	1.76E+08	2.02E+08	2.26E+08	1.90E+08

Appendix 5C – All proteins detected on S-HDL-P in women and men

198 proteins were detected on S-HDL-P in women and men and are shown below.

Table 5.12 *Proteins detected on S-HDL-P in women and men with and without T1DM or T2DM.*

Protein names	Majority protein IDs	Non-OB	T1DM	OB	T2DM	Non-OB	T1DM	OB	T2DM
	Uniprot	Mean LFQ Intensity Women				Mean LFQ Intensity Men			
72 kDa type IV collagenase	P08253	1.54E+07	1.35E+07	1.70E+07	1.20E+07	1.24E+07	1.54E+07	1.68E+07	1.27E+07
A-kinase anchor protein 9	A0A2R8Y590	1.95E+08	1.79E+08	1.50E+08	1.42E+08	1.40E+08	1.47E+08	1.47E+08	1.31E+08
Actin, cytoplasmic 1	P60709	2.75E+08	2.35E+08	2.56E+08	2.49E+08	2.84E+08	2.66E+08	3.55E+08	3.22E+08
Adipocyte plasma membrane-associated protein	Q9HDC9	6.26E+07	5.15E+07	3.56E+07	3.58E+07	3.62E+07	5.42E+07	3.97E+07	7.38E+07
Afamin	P43652	9.20E+09	7.73E+09	8.75E+09	8.94E+09	8.99E+09	7.36E+09	1.03E+10	1.01E+10
Albumin	P02768	3.12E+11	3.02E+11	2.69E+11	2.58E+11	2.84E+11	2.68E+11	2.46E+11	2.24E+11
Alpha-1-acid glycoprotein 1	P02763	4.77E+07	4.24E+07	4.56E+07	4.81E+07	3.87E+07	3.85E+07	3.66E+07	3.63E+07
Alpha-1-acid glycoprotein 2	P19652	9.82E+07	1.80E+08	1.00E+08	1.01E+08	1.53E+08	9.94E+07	9.87E+07	1.21E+08
Alpha-1-antichymotrypsin	P01011	1.46E+10	1.43E+10	1.24E+10	1.12E+10	1.10E+10	1.30E+10	1.11E+10	1.03E+10
Alpha-1-antitrypsin	P01009	8.45E+09	7.72E+09	9.73E+09	5.92E+09	8.06E+09	5.63E+09	4.51E+09	4.32E+09
Alpha-1B-glycoprotein	P04217	1.71E+10	1.89E+10	1.71E+10	1.65E+10	1.50E+10	1.87E+10	1.60E+10	1.60E+10
Alpha-2-antiplasmin	P08697	3.20E+09	3.56E+09	3.11E+09	3.10E+09	3.09E+09	3.47E+09	2.61E+09	3.49E+09
Alpha-2-HS-glycoprotein	P02765	1.04E+10	9.18E+09	9.01E+09	1.06E+10	9.58E+09	8.34E+09	9.54E+09	1.08E+10
Alpha-2-macroglobulin	P01023	5.62E+08	5.97E+08	4.99E+08	3.80E+08	3.87E+08	3.99E+08	2.68E+08	3.11E+08
Angiotensinogen	A0A7P0T9S6	9.90E+08	1.01E+09	1.14E+09	1.13E+09	6.86E+08	8.73E+08	7.63E+08	8.83E+08
Antithrombin-III	P01008	7.06E+08	8.28E+08	6.50E+08	5.07E+08	6.94E+08	4.78E+08	3.62E+08	4.27E+08
Apolipoprotein A-I	P02647	1.51E+11	1.40E+11	1.40E+11	1.58E+11	1.66E+11	1.62E+11	1.61E+11	1.55E+11
Apolipoprotein A-II	P02652	2.10E+10	1.66E+10	2.01E+10	2.35E+10	2.30E+10	2.13E+10	2.61E+10	2.15E+10
Apolipoprotein A-IV	P06727	4.95E+09	6.10E+09	4.48E+09	5.92E+09	4.34E+09	6.05E+09	4.53E+09	6.69E+09
Apolipoprotein B-100	P04114	1.62E+08	1.07E+08	1.55E+08	1.31E+08	1.42E+08	1.04E+08	1.29E+08	1.63E+08
Apolipoprotein C-I (Fragment)	K7ERI9	1.27E+09	1.22E+09	1.42E+09	1.48E+09	1.63E+09	1.71E+09	1.57E+09	2.22E+09

Apolipoprotein C-II	K7ER74	3.20E+08	2.03E+08	4.68E+08	4.61E+08	5.26E+08	4.08E+08	4.91E+08	9.22E+08
Apolipoprotein C-III	P02656	2.03E+09	1.84E+09	2.76E+09	2.72E+09	3.12E+09	2.48E+09	3.40E+09	5.22E+09
Apolipoprotein C-IV	P55056	7.95E+07	6.16E+07	1.03E+08	9.29E+07	1.31E+08	8.80E+07	8.11E+07	1.25E+08
Apolipoprotein D	P05090	3.11E+09	2.38E+09	2.78E+09	2.92E+09	4.74E+09	4.00E+09	3.89E+09	3.30E+09
Apolipoprotein E	P02649	1.28E+09	1.09E+09	1.52E+09	1.31E+09	1.43E+09	1.50E+09	1.46E+09	2.33E+09
Apolipoprotein L1	O14791	1.12E+08	9.73E+07	2.71E+08	2.48E+08	1.98E+08	1.03E+08	1.63E+08	3.50E+08
Apolipoprotein M	O95445	1.66E+09	1.80E+09	2.02E+09	1.99E+09	2.30E+09	2.32E+09	2.27E+09	2.48E+09
Beta-2-glycoprotein 1	P02749	2.13E+09	1.53E+09	2.14E+09	3.08E+09	7.93E+08	2.25E+09	1.32E+09	7.14E+08
Beta-Ala-His dipeptidase	Q96KN2	3.21E+08	3.09E+08	3.41E+08	3.17E+08	3.22E+08	3.10E+08	3.28E+08	3.81E+08
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	Q9H3R1	8.60E+08	1.05E+09	6.70E+08	6.92E+08	8.12E+08	9.74E+08	7.48E+08	1.08E+09
Biotinidase	P43251	1.77E+08	1.77E+08	1.57E+08	1.75E+08	1.40E+08	1.56E+08	1.92E+08	1.81E+08
C3/C5 convertase	B4E1Z4	3.12E+10	3.17E+10	3.68E+10	4.02E+10	2.96E+10	3.59E+10	3.25E+10	3.99E+10
C4b-binding protein alpha chain	P04003	1.24E+08	1.85E+08	1.21E+08	1.21E+08	1.10E+08	1.21E+08	1.10E+08	1.41E+08
CD5 antigen-like	O43866	4.95E+07	4.50E+07	4.16E+07	5.02E+07	3.53E+07	5.34E+07	5.13E+07	4.44E+07
Ceruloplasmin	P00450	6.04E+10	7.31E+10	6.37E+10	5.96E+10	5.31E+10	6.03E+10	5.50E+10	5.34E+10
Clusterin	P10909	1.43E+10	1.32E+10	1.40E+10	1.54E+10	1.37E+10	1.54E+10	1.55E+10	1.69E+10
Coagulation factor IX	P00740	5.78E+08	5.29E+08	6.49E+08	6.58E+08	5.17E+08	6.77E+08	5.28E+08	8.68E+08
Coagulation factor V	P12259	1.17E+08	9.34E+07	1.18E+08	1.30E+08	1.26E+08	1.39E+08	1.08E+08	1.28E+08
Coagulation factor X	P00742	8.67E+08	7.75E+08	8.49E+08	8.72E+08	9.75E+08	1.05E+09	1.04E+09	1.07E+09
Coagulation factor XII	P00748	2.10E+08	2.30E+08	2.45E+08	2.47E+08	2.16E+08	1.81E+08	2.30E+08	2.22E+08
Coagulation factor XIII B chain	P05160	1.29E+09	1.26E+09	1.33E+09	9.98E+08	1.31E+09	1.30E+09	1.38E+09	1.21E+09
Complement C1r subcomponent-like protein	Q9NZP8	2.60E+08	2.74E+08	2.70E+08	3.10E+08	2.83E+08	2.99E+08	2.62E+08	3.17E+08
Complement C1s subcomponent	P09871	1.06E+09	8.16E+08	1.23E+09	1.09E+09	1.17E+09	1.11E+09	1.12E+09	1.08E+09
Complement C2	P06681	3.54E+08	4.26E+08	3.92E+08	3.88E+08	3.58E+08	4.48E+08	3.47E+08	4.33E+08
Complement C3	P01024	3.14E+11	3.05E+11	3.45E+11	3.65E+11	3.49E+11	3.48E+11	4.01E+11	4.14E+11
Complement C4-A	P0C0L4	6.00E+08	9.19E+08	1.06E+09	9.21E+08	8.88E+08	5.88E+08	6.66E+08	6.91E+08
Complement C4-B	P0C0L5	2.86E+10	3.68E+10	3.29E+10	3.32E+10	2.95E+10	2.52E+10	3.51E+10	2.84E+10
Complement C5	P01031	9.33E+09	1.01E+10	1.11E+10	1.12E+10	9.82E+09	1.09E+10	1.20E+10	1.30E+10

Complement component C6	P13671	2.26E+09	2.31E+09	2.76E+09	2.56E+09	2.24E+09	2.35E+09	2.52E+09	2.31E+09
Complement component C7	P10643	2.27E+09	2.70E+09	2.17E+09	2.29E+09	2.63E+09	2.77E+09	2.26E+09	2.96E+09
Complement component C8 alpha chain	P07357	1.27E+09	1.38E+09	1.42E+09	1.47E+09	8.97E+08	1.34E+09	9.02E+08	1.23E+09
Complement component C8 beta chain	P07358	1.53E+09	1.48E+09	1.73E+09	1.67E+09	1.06E+09	1.70E+09	1.24E+09	1.58E+09
Complement component C8 gamma chain	P07360	1.01E+09	9.92E+08	1.22E+09	1.20E+09	8.14E+08	1.09E+09	7.74E+08	9.53E+08
Complement component C9	P02748	2.97E+09	2.81E+09	3.51E+09	3.01E+09	1.92E+09	3.18E+09	2.07E+09	2.45E+09
Complement factor H	P08603	1.09E+09	1.07E+09	1.18E+09	1.17E+09	1.05E+09	1.16E+09	1.10E+09	1.25E+09
Complement factor H-related protein 1	Q03591	2.62E+09	2.61E+09	2.72E+09	2.39E+09	2.29E+09	2.77E+09	2.34E+09	2.77E+09
Complement factor H-related protein 2	P36980	8.29E+08	6.06E+08	7.01E+08	5.57E+08	6.69E+08	7.73E+08	6.04E+08	9.33E+08
Complement factor I	G3XAM2	1.58E+09	1.50E+09	1.68E+09	1.75E+09	1.24E+09	1.49E+09	1.53E+09	1.67E+09
Complement subcomponent C1r	A0A3B3ISR2	1.40E+08	1.58E+08	1.50E+08	2.90E+08	1.03E+08	1.42E+08	1.48E+08	1.54E+08
Cysteine-rich secretory protein 3	P54108	5.94E+07	5.55E+07	4.32E+07	4.18E+07	5.36E+07	4.99E+07	4.69E+07	4.09E+07
EGF-containing fibulin-like extracellular matrix protein 1	Q12805	1.07E+08	1.11E+08	1.29E+08	9.26E+07	1.15E+08	1.26E+08	1.03E+08	1.15E+08
Endoplasmic reticulum chaperone BiP	P11021	4.61E+07	4.61E+07	4.54E+07	5.19E+07	4.14E+07	5.17E+07	4.48E+07	6.52E+07
Extracellular matrix protein 1	Q16610	3.84E+08	4.02E+08	3.70E+08	3.63E+08	4.37E+08	3.84E+08	3.74E+08	4.40E+08
Fibrinogen alpha chain	P02671	4.23E+07	2.97E+07	3.99E+07	4.03E+07	2.42E+07	3.28E+07	4.37E+07	3.80E+07
Fibronectin	P02751	5.16E+07	6.71E+07	3.95E+07	4.28E+07	5.68E+07	3.62E+07	3.32E+07	5.12E+07
Fibulin-1	P23142	1.86E+07	7.79E+06	1.01E+07	1.41E+07	1.05E+07	1.04E+07	1.03E+07	8.11E+06
Fructose-bisphosphate aldolase	A0A3B3IS80	2.09E+07	1.79E+07	1.73E+07	5.49E+07	2.88E+07	3.13E+07	5.14E+07	4.58E+07
Gelsolin	P06396	1.16E+09	1.04E+09	1.06E+09	9.99E+08	1.04E+09	1.01E+09	1.02E+09	8.94E+08
Glutathione peroxidase	A0A087X1J7	1.80E+08	1.62E+08	1.85E+08	1.69E+08	1.44E+08	1.83E+08	1.64E+08	2.00E+08
Haptoglobin	P00738	8.00E+09	6.65E+09	4.44E+09	9.17E+09	5.09E+09	1.45E+10	9.02E+09	3.55E+09
Haptoglobin-related protein	P00739	4.66E+07	4.43E+07	4.11E+07	4.17E+07	4.25E+07	7.06E+07	3.80E+07	3.03E+07
Hemoglobin subunit alpha	P69905	7.83E+07	1.03E+08	8.30E+07	1.05E+08	1.05E+08	1.07E+08	7.62E+07	1.90E+08
Hemoglobin subunit beta	P68871	3.54E+07	3.78E+07	8.68E+07	7.85E+07	2.59E+07	7.40E+07	2.13E+07	1.37E+08
Hemopexin	P02790	5.05E+10	4.63E+10	4.72E+10	4.91E+10	4.35E+10	4.31E+10	4.04E+10	4.37E+10
Heparin cofactor 2	P05546	6.91E+09	7.04E+09	7.91E+09	8.22E+09	5.36E+09	7.19E+09	7.14E+09	8.28E+09
Hepatocyte growth factor activator	Q04756	1.44E+08	1.52E+08	1.47E+08	1.39E+08	1.33E+08	1.12E+08	1.51E+08	1.54E+08

Histidine-rich glycoprotein	P04196	3.37E+09	3.47E+09	3.40E+09	3.69E+09	3.60E+09	3.28E+09	3.33E+09	4.07E+09
Hyaluronan-binding protein 2	Q14520	8.64E+07	7.56E+07	7.41E+07	7.87E+07	4.46E+07	6.32E+07	5.15E+07	7.73E+07
Ig-like domain-containing protein (Fragment)	A0A0G2JRQ6	3.55E+08	4.39E+08	3.47E+08	4.79E+08	4.32E+08	4.84E+08	3.45E+08	4.25E+08
Ig-like domain-containing protein (Fragment)	A0A0J9YY99	6.68E+09	7.80E+09	6.32E+09	7.12E+09	7.02E+09	6.64E+09	8.81E+09	5.66E+09
Immunoglobulin heavy constant alpha 1	P01876	9.25E+09	1.05E+10	9.36E+09	9.01E+09	1.25E+10	1.01E+10	9.01E+09	9.07E+09
Immunoglobulin heavy constant alpha 2 (Fragment)	A0A0G2JMB2	3.02E+08	4.48E+08	4.75E+08	3.22E+08	4.73E+08	3.64E+08	3.02E+08	3.00E+08
Immunoglobulin heavy constant gamma 1	P01857	9.48E+10	9.73E+10	9.26E+10	8.20E+10	1.11E+11	9.37E+10	1.00E+11	7.48E+10
Immunoglobulin heavy constant gamma 2	P01859	4.61E+10	4.41E+10	4.43E+10	4.65E+10	4.49E+10	4.51E+10	4.64E+10	4.01E+10
Immunoglobulin heavy constant gamma 3	P01860	2.18E+10	2.47E+10	2.20E+10	2.32E+10	2.41E+10	2.11E+10	2.06E+10	1.95E+10
Immunoglobulin heavy constant gamma 4	P01861	7.03E+09	7.36E+09	5.94E+09	5.39E+09	1.14E+10	8.76E+09	1.04E+10	9.34E+09
Immunoglobulin heavy constant mu	P01871	6.86E+08	7.13E+08	5.81E+08	4.91E+08	5.65E+08	5.32E+08	5.43E+08	7.24E+08
Immunoglobulin heavy variable 1-18	A0A0C4DH31	5.26E+08	4.78E+08	5.15E+08	4.13E+08	4.14E+08	4.11E+08	5.41E+08	3.74E+08
Immunoglobulin heavy variable 1-2	P23083	2.36E+09	2.09E+09	1.93E+09	1.44E+09	1.63E+09	1.56E+09	2.06E+09	1.94E+09
Immunoglobulin heavy variable 1-24	A0A0C4DH33	6.63E+07	1.01E+08	7.11E+07	6.59E+07	6.78E+07	6.64E+07	5.64E+07	4.88E+07
Immunoglobulin heavy variable 1-46	P01743	2.06E+08	1.52E+08	1.06E+08	1.30E+08	1.47E+08	1.39E+08	1.18E+08	1.41E+08
Immunoglobulin heavy variable 1-69	P01742	3.00E+09	2.65E+09	2.37E+09	2.35E+09	2.11E+09	2.28E+09	2.53E+09	2.45E+09
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	A0A075B7D0	1.17E+09	1.87E+09	1.73E+09	1.11E+09	1.68E+09	1.84E+09	1.54E+09	1.04E+09
Immunoglobulin heavy variable 2-26	A0A0B4J1V2	2.45E+08	3.17E+08	2.21E+08	2.78E+08	3.49E+08	3.94E+08	2.48E+08	1.81E+08
Immunoglobulin heavy variable 2-70D	A0A0C4DH43	1.04E+09	7.75E+08	6.43E+08	5.29E+08	7.04E+08	6.06E+08	6.89E+08	5.95E+08
Immunoglobulin heavy variable 3-13	P01766	5.36E+08	4.46E+08	4.23E+08	4.00E+08	4.83E+08	4.12E+08	5.23E+08	3.00E+08
Immunoglobulin heavy variable 3-15	A0A0B4J1V0	1.01E+09	8.77E+08	8.43E+08	7.50E+08	6.47E+08	7.94E+08	9.07E+08	7.59E+08
Immunoglobulin heavy variable 3-43	A0A0B4J1X8	1.83E+08	1.48E+08	1.34E+08	1.10E+08	1.86E+08	2.33E+08	1.29E+08	1.17E+08
Immunoglobulin heavy variable 3-49	A0A0A0MS15	6.31E+08	8.03E+08	7.80E+08	8.49E+08	9.50E+08	8.53E+08	1.12E+09	7.64E+08
Immunoglobulin heavy variable 3-7	P01780	1.09E+10	1.25E+10	1.14E+10	1.19E+10	9.07E+09	9.69E+09	1.18E+10	8.79E+09
Immunoglobulin heavy variable 3-72	A0A4W8ZXM2	1.12E+10	1.25E+10	1.20E+10	1.06E+10	1.05E+10	9.71E+09	1.30E+10	9.60E+09
Immunoglobulin heavy variable 3-73	A0A0B4J1V6	6.09E+08	7.00E+08	5.10E+08	4.88E+08	4.37E+08	3.20E+08	6.28E+08	4.26E+08
Immunoglobulin heavy variable 3-74	A0A0B4J1X5	1.48E+09	1.72E+09	1.32E+09	1.53E+09	1.05E+09	1.34E+09	1.88E+09	1.12E+09
Immunoglobulin heavy variable 3-9	P01782	2.22E+09	1.74E+09	1.64E+09	1.75E+09	2.12E+09	1.81E+09	1.43E+09	1.42E+09

Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Fragment)	A0A075B7B8	2.17E+09	2.48E+09	2.17E+09	1.13E+09	2.72E+09	2.67E+09	2.48E+09	1.34E+09
Immunoglobulin heavy variable 4-28	A0A0C4DH34	1.93E+09	1.72E+09	1.76E+09	1.46E+09	1.81E+09	1.66E+09	1.81E+09	1.40E+09
Immunoglobulin heavy variable 4-30-2	A0A087WSY4	2.28E+08	2.54E+08	2.25E+08	2.78E+08	2.66E+08	3.17E+08	2.66E+08	2.36E+08
Immunoglobulin heavy variable 4-38-2	P0DP08	3.45E+09	4.09E+09	2.93E+09	2.90E+09	2.89E+09	3.35E+09	3.68E+09	2.72E+09
Immunoglobulin heavy variable 4-4	A0A075B6R2	6.41E+08	1.06E+09	7.68E+08	8.25E+08	7.38E+08	7.02E+08	7.15E+08	5.90E+08
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	A0A075B7B6	3.63E+08	4.94E+08	3.85E+08	3.51E+08	3.49E+08	4.74E+08	5.28E+08	2.88E+08
Immunoglobulin heavy variable 5-51	A0A0C4DH38	3.11E+09	3.17E+09	2.75E+09	2.82E+09	2.97E+09	2.90E+09	3.32E+09	2.38E+09
Immunoglobulin heavy variable 6-1	A0A0B4J1U7	1.55E+09	1.23E+09	1.33E+09	1.22E+09	1.22E+09	1.33E+09	1.08E+09	1.29E+09
Immunoglobulin J chain	P01591	5.24E+07	5.42E+07	4.84E+07	4.96E+07	3.63E+07	5.69E+07	5.39E+07	5.27E+07
Immunoglobulin kappa constant	P01834	4.37E+10	4.14E+10	4.33E+10	3.96E+10	5.24E+10	4.45E+10	4.70E+10	3.66E+10
Immunoglobulin kappa variable 1-17	P01599	3.82E+08	5.03E+08	4.16E+08	4.37E+08	3.73E+08	3.59E+08	4.72E+08	3.40E+08
Immunoglobulin kappa variable 1-33	A0A2Q2TTZ9	4.72E+09	5.40E+09	4.19E+09	4.36E+09	5.47E+09	5.10E+09	5.14E+09	4.07E+09
Immunoglobulin kappa variable 1-5	P01602	1.60E+09	1.68E+09	1.67E+09	1.70E+09	1.76E+09	2.05E+09	2.03E+09	1.48E+09
Immunoglobulin kappa variable 1-6	A0A0C4DH72	2.34E+08	1.43E+08	1.24E+08	1.28E+08	1.55E+08	1.59E+08	1.82E+08	1.19E+08
Immunoglobulin kappa variable 1-8	A0A0C4DH67	2.39E+08	2.72E+08	2.22E+08	2.15E+08	2.50E+08	2.00E+08	2.46E+08	1.81E+08
Immunoglobulin kappa variable 1D-39	P04432	7.21E+07	5.68E+07	7.76E+07	5.53E+07	5.86E+07	5.33E+07	6.67E+07	5.77E+07
Immunoglobulin kappa variable 2-24	A0A0C4DH68	1.82E+09	1.81E+09	1.46E+09	1.23E+09	1.67E+09	1.16E+09	1.58E+09	1.11E+09
Immunoglobulin kappa variable 2-30	P06310	1.32E+09	1.29E+09	1.41E+09	1.19E+09	9.77E+08	9.09E+08	1.20E+09	8.31E+08
Immunoglobulin kappa variable 2-40	A0A087X0Q4	8.32E+09	7.03E+09	7.28E+09	6.00E+09	7.94E+09	7.41E+09	8.79E+09	6.46E+09
Immunoglobulin kappa variable 2D-28	A0A5H1ZRS2	1.19E+09	1.08E+09	9.79E+08	7.20E+08	1.30E+09	9.50E+08	1.18E+09	7.63E+08
Immunoglobulin kappa variable 2D-29	A0A5H1ZRS9	5.45E+08	8.09E+08	1.01E+09	5.92E+08	7.07E+08	6.28E+08	9.70E+08	6.27E+08
Immunoglobulin kappa variable 3-11	P04433	5.41E+09	5.13E+09	4.27E+09	3.90E+09	5.67E+09	4.54E+09	5.09E+09	5.17E+09
Immunoglobulin kappa variable 3-20	P01619	6.94E+09	6.44E+09	6.47E+09	5.96E+09	6.30E+09	6.44E+09	7.65E+09	5.36E+09
Immunoglobulin kappa variable 3D-15	A0A087WSY6	6.86E+08	6.67E+08	5.78E+08	5.70E+08	6.06E+08	6.64E+08	7.43E+08	5.29E+08
Immunoglobulin kappa variable 4-1	P06312	2.68E+09	3.17E+09	2.76E+09	2.79E+09	3.13E+09	3.06E+09	3.46E+09	2.44E+09
Immunoglobulin kappa variable 6-21	A0A0C4DH24	3.39E+08	6.34E+08	3.41E+08	3.86E+08	4.99E+08	4.08E+08	3.46E+08	3.11E+08
Immunoglobulin lambda constant 2	P0DOY2	1.05E+07	1.30E+07	1.22E+07	1.27E+07	2.23E+07	8.39E+06	6.80E+07	8.75E+06
Immunoglobulin lambda constant 3	P0DOY3	2.68E+10	2.55E+10	2.45E+10	2.49E+10	2.79E+10	2.29E+10	2.76E+10	2.11E+10

Immunoglobulin lambda constant 7 (Fragment)	A0A5H1ZRQ7	1.21E+08	1.59E+08	1.08E+08	9.66E+07	1.42E+08	1.63E+08	1.42E+08	1.33E+08
Immunoglobulin lambda variable 1-40	P01703	2.09E+08	2.16E+08	1.69E+08	1.85E+08	1.94E+08	1.51E+08	1.73E+08	1.13E+08
Immunoglobulin lambda variable 1-47	P01700	1.85E+09	2.51E+09	1.88E+09	2.42E+09	1.84E+09	2.11E+09	2.22E+09	1.88E+09
Immunoglobulin lambda variable 1-51	P01701	2.69E+08	3.55E+08	2.43E+08	2.28E+08	2.86E+08	2.81E+08	2.59E+08	1.97E+08
Immunoglobulin lambda variable 2-11	P01706	3.31E+08	3.33E+08	2.51E+08	2.88E+08	2.98E+08	3.00E+08	2.58E+08	2.65E+08
Immunoglobulin lambda variable 2-14	P01704	4.34E+08	2.35E+08	1.70E+08	2.92E+08	3.33E+08	2.71E+08	3.01E+08	1.68E+08
Immunoglobulin lambda variable 2-18	A0A075B6J9	2.34E+08	2.14E+08	1.10E+08	1.42E+08	1.13E+08	1.69E+08	1.39E+08	1.25E+08
Immunoglobulin lambda variable 3-10	A0A075B6K4	1.02E+09	1.13E+09	1.10E+09	1.13E+09	1.38E+09	1.25E+09	1.36E+09	9.74E+08
Immunoglobulin lambda variable 3-16	A0A075B6K0	6.34E+08	5.53E+08	4.53E+08	4.36E+08	4.96E+08	4.81E+08	5.29E+08	3.64E+08
Immunoglobulin lambda variable 3-19	P01714	1.39E+09	1.38E+09	9.69E+08	9.50E+08	1.30E+09	1.14E+09	8.91E+08	1.06E+09
Immunoglobulin lambda variable 3-21	P80748	9.73E+07	1.23E+08	1.30E+08	1.34E+08	1.76E+08	1.59E+08	1.55E+08	1.17E+08
Immunoglobulin lambda variable 3-9	A0A075B6K5	1.23E+09	1.37E+09	1.07E+09	1.15E+09	1.56E+09	1.33E+09	1.21E+09	1.08E+09
Immunoglobulin lambda variable 4-60	A0A075B6I1	3.00E+07	3.69E+07	2.42E+07	3.01E+07	4.23E+07	3.47E+07	4.43E+07	3.54E+07
Immunoglobulin lambda variable 4-69	A0A075B6H9	2.51E+08	2.89E+08	1.71E+08	2.54E+08	2.40E+08	1.87E+08	2.67E+08	2.01E+08
Immunoglobulin lambda variable 5-45 (Fragment)	A0A0G2JSC0	5.96E+07	6.81E+07	3.13E+07	5.23E+07	5.21E+07	5.38E+07	5.98E+07	4.83E+07
Immunoglobulin lambda variable 7-46	A0A075B6I9	6.35E+08	7.94E+08	5.70E+08	6.77E+08	9.29E+08	8.12E+08	6.92E+08	6.19E+08
Immunoglobulin lambda variable 8-61	A0A075B6I0	7.83E+08	6.81E+08	3.76E+08	5.53E+08	4.45E+08	7.77E+08	7.24E+08	5.67E+08
Immunoglobulin lambda variable 9-49	A0A0B4J1Y8	7.94E+07	2.20E+08	8.29E+07	1.15E+08	9.35E+07	1.12E+08	1.39E+08	1.47E+08
Immunoglobulin lambda-like polypeptide 1	P15814	1.47E+08	1.11E+08	1.65E+08	9.63E+07	1.41E+08	1.30E+08	7.72E+07	8.76E+07
Immunoglobulin lambda-like polypeptide 5	B9A064	4.06E+09	4.65E+09	4.22E+09	3.87E+09	4.64E+09	3.97E+09	4.33E+09	3.88E+09
Insulin-like growth factor-binding protein 3	A6XND1	5.83E+08	3.68E+08	5.21E+08	5.96E+08	5.17E+08	4.65E+08	5.32E+08	5.74E+08
Insulin-like growth factor-binding protein complex acid labile subunit	P35858	3.04E+09	2.76E+09	2.76E+09	3.19E+09	2.87E+09	2.45E+09	2.92E+09	2.30E+09
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	5.99E+08	1.01E+09	5.68E+08	5.10E+08	5.97E+08	5.04E+08	4.50E+08	4.70E+08
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	7.60E+08	1.10E+09	6.97E+08	5.89E+08	8.02E+08	5.34E+08	5.66E+08	5.05E+08
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033	9.77E+07	1.47E+08	9.66E+07	7.85E+07	4.93E+07	7.67E+07	4.23E+07	5.18E+07
ITIH4 protein	B7ZKJ8	2.05E+10	1.91E+10	2.28E+10	2.17E+10	2.17E+10	2.42E+10	2.05E+10	2.29E+10
Kallistatin	P29622	3.05E+07	3.78E+07	3.53E+07	3.42E+07	3.04E+07	3.25E+07	3.13E+07	3.12E+07
Keratin, type I cytoskeletal 10	P13645	6.79E+07	8.84E+07	4.89E+07	4.92E+07	5.14E+07	4.20E+07	4.35E+07	8.18E+07

Keratin, type I cytoskeletal 9	P35527	1.08E+08	6.19E+07	6.06E+07	1.23E+08	7.64E+07	5.61E+07	8.32E+07	2.34E+08
Keratin, type II cytoskeletal 1	P04264	2.69E+08	1.15E+08	1.42E+08	1.30E+08	1.57E+08	1.01E+08	1.49E+08	2.20E+08
Kininogen-1	P01042	1.90E+10	2.47E+10	2.26E+10	2.48E+10	1.89E+10	1.92E+10	2.08E+10	2.31E+10
L-lactate dehydrogenase B chain	P07195	4.49E+07	5.39E+07	5.06E+07	4.82E+07	4.40E+07	4.89E+07	5.80E+07	6.15E+07
L-selectin	P14151	1.41E+08	1.36E+08	1.24E+08	1.17E+08	1.71E+08	1.40E+08	1.28E+08	1.34E+08
Low affinity immunoglobulin gamma Fc region receptor III-A	P08637	3.75E+07	3.32E+07	3.61E+07	3.29E+07	5.48E+07	3.11E+07	4.00E+07	4.70E+07
Lumican	P51884	2.93E+09	3.11E+09	2.85E+09	2.75E+09	2.93E+09	3.55E+09	3.04E+09	3.12E+09
N-acetylmuramoyl-L-alanine amidase	Q96PD5	1.44E+09	1.30E+09	1.17E+09	1.25E+09	1.18E+09	1.17E+09	1.33E+09	1.14E+09
Phosphatidylcholine-sterol acyltransferase	P04180	6.09E+07	6.40E+07	6.03E+07	6.14E+07	7.22E+07	5.70E+07	7.47E+07	7.26E+07
Phosphatidylinositol-glycan-specific phospholipase D	P80108	7.38E+07	2.06E+08	2.05E+08	1.04E+08	1.43E+08	1.01E+08	1.29E+08	1.72E+08
Plasma kallikrein	P03952	7.75E+08	8.85E+08	9.55E+08	1.06E+09	7.92E+08	8.22E+08	8.72E+08	1.59E+09
Plasma protease C1 inhibitor	A0A7I2V2D2	2.74E+08	2.29E+08	3.44E+08	3.42E+08	2.62E+08	2.82E+08	2.95E+08	2.94E+08
Plasminogen	P00747	4.61E+08	4.39E+08	5.73E+08	5.79E+08	3.06E+08	3.71E+08	3.34E+08	4.53E+08
Platelet factor 4	P02776	6.54E+07	8.36E+07	6.62E+07	6.41E+07	7.43E+07	7.26E+07	6.62E+07	9.29E+07
Probable non-functional immunoglobulin heavy variable 3-35	A0A0C4DH35	4.29E+08	5.10E+08	3.86E+08	3.32E+08	2.99E+08	3.61E+08	3.90E+08	4.06E+08
Probable non-functional immunoglobulin heavy variable 3-38	A0A0C4DH36	2.95E+08	2.36E+08	2.20E+08	1.77E+08	2.37E+08	2.50E+08	2.68E+08	2.00E+08
Protein AMBP	P02760	7.63E+08	8.29E+08	7.81E+08	6.82E+08	8.52E+08	8.34E+08	7.51E+08	8.19E+08
Protein Z-dependent protease inhibitor	Q9UK55	4.75E+08	3.81E+08	4.60E+08	4.14E+08	3.83E+08	4.44E+08	4.86E+08	5.49E+08
Prothrombin	P00734	8.61E+09	1.33E+10	9.25E+09	9.30E+09	1.28E+10	1.11E+10	8.26E+09	1.21E+10
SAA2-SAA4 readthrough	A0A096LPE2	4.80E+09	4.21E+09	5.08E+09	6.34E+09	5.10E+09	5.20E+09	5.58E+09	5.49E+09
Selenoprotein P	P49908	2.93E+08	2.41E+08	2.80E+08	3.55E+08	2.77E+08	2.85E+08	2.74E+08	3.74E+08
Serotransferrin	P02787	2.50E+10	2.42E+10	2.29E+10	2.38E+10	1.93E+10	2.06E+10	1.84E+10	2.09E+10
Serum amyloid P-component	P02743	1.40E+09	9.83E+08	1.75E+09	1.49E+09	1.46E+09	1.56E+09	1.63E+09	2.13E+09
Serum paraoxonase/arylesterase 1	P27169	3.18E+09	2.65E+09	3.21E+09	3.27E+09	3.52E+09	2.99E+09	3.22E+09	3.37E+09
Sex hormone-binding globulin	I3L145	4.27E+08	6.01E+08	2.80E+08	2.60E+08	2.88E+08	2.78E+08	2.10E+08	1.31E+08
SPARC	P09486	2.68E+08	2.79E+08	3.06E+08	2.92E+08	3.31E+08	3.44E+08	3.47E+08	2.82E+08
Sulfhydryl oxidase 1	O00391	1.40E+08	1.38E+08	1.45E+08	1.47E+08	1.72E+08	1.57E+08	1.54E+08	1.69E+08
Tetranectin	E9PHK0	2.21E+09	1.93E+09	2.43E+09	1.90E+09	2.85E+09	2.27E+09	2.64E+09	2.77E+09

Thyroxine-binding globulin	P05543	3.63E+07	2.92E+07	4.21E+07	3.08E+07	3.02E+07	2.93E+07	3.41E+07	3.45E+07
Transferrin receptor protein 1	P02786	4.49E+07	4.28E+07	4.08E+07	4.52E+07	2.86E+07	2.77E+07	2.28E+07	3.75E+07
Transforming growth factor-beta-induced protein ig-h3	Q15582	2.42E+08	2.14E+08	2.26E+08	2.21E+08	2.27E+08	2.39E+08	2.80E+08	2.73E+08
Transthyretin	A0A087WT59	6.85E+07	5.34E+07	8.54E+07	4.96E+07	1.09E+08	3.76E+07	5.38E+07	4.93E+07
Vitamin D-binding protein	P02774	3.59E+08	3.30E+08	3.73E+08	3.24E+08	3.42E+08	3.42E+08	3.60E+08	3.90E+08
Vitamin K-dependent protein C	P04070	3.74E+08	3.29E+08	3.10E+08	3.36E+08	3.57E+08	3.81E+08	3.28E+08	4.21E+08
Vitamin K-dependent protein S	A0A3B3ISJ1	5.28E+08	5.99E+08	5.72E+08	5.85E+08	6.47E+08	6.96E+08	7.08E+08	7.22E+08
Vitamin K-dependent protein Z	P22891	4.53E+07	4.93E+07	4.65E+07	4.62E+07	6.15E+07	6.63E+07	6.82E+07	4.07E+07
Vitronectin	P04004	8.89E+09	8.33E+09	9.77E+09	1.04E+10	7.86E+09	8.60E+09	8.90E+09	1.03E+10

Appendix 5D – Modulation of the proteomic composition of L-HDL-P

I. *Modulation of the proteomic composition of L-HDL-P in women vs. men without obesity*

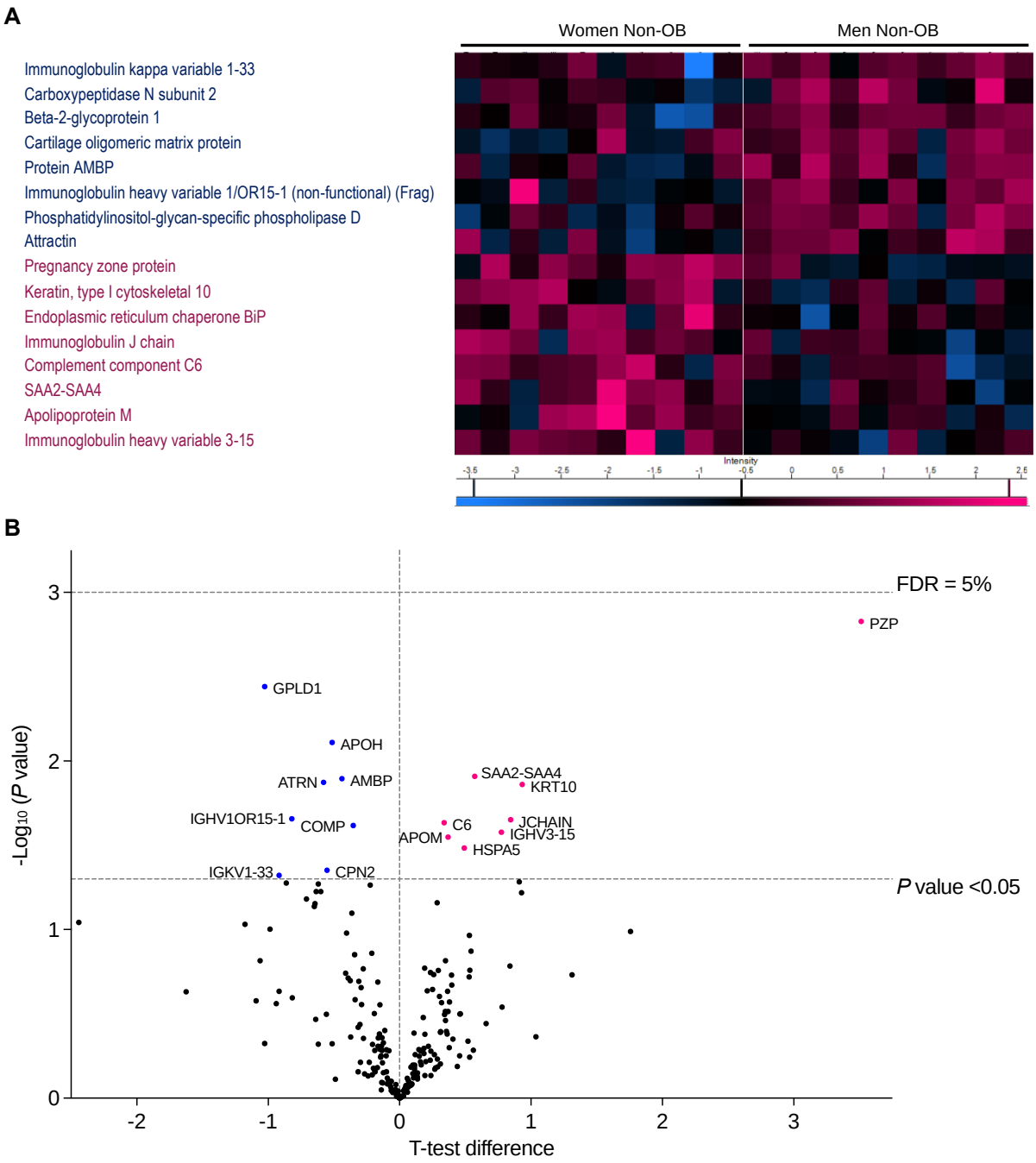

Figure 5.9 *Significantly changed proteins on large HDL-P between women and men without obesity.*
HDL was isolated via FPLC from women and men without obesity (*n*=10 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between men and women were identified by mass spectrometry.
(A) Perseus software was used to determine significantly (*P*<0.05) changed proteins and presented in a heatmap.
(B) Volcano plot showing the differences in protein abundance detected between women and men without obesity. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

II. Modulation of the proteomic composition of L-HDL-P in women vs. men with T1DM

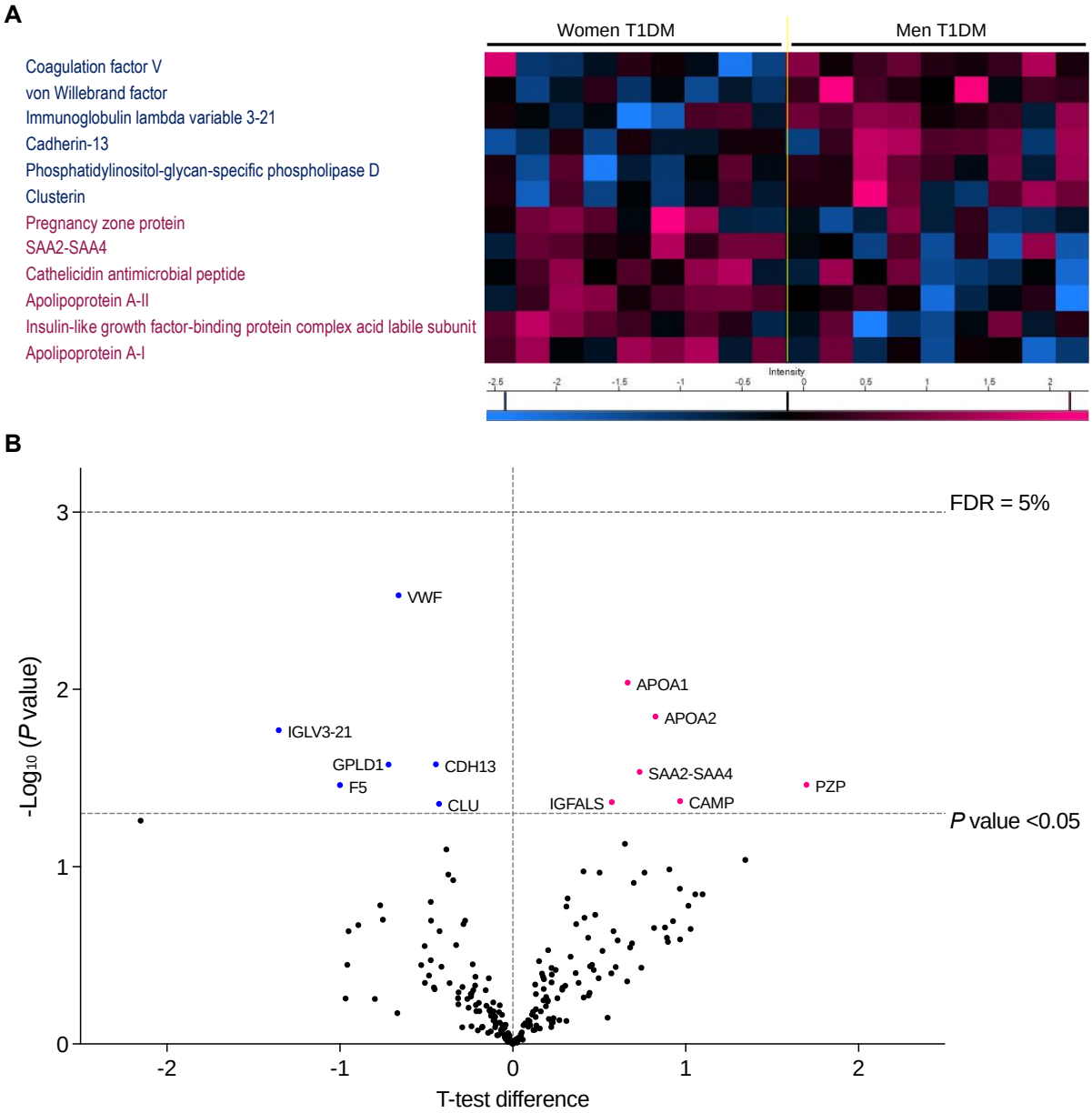

Figure 5.10 *Significantly changed proteins on large HDL-P between women and men with T1DM.*
HDL was isolated via FPLC from women and men with T1DM (*n*=10 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between men and women were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (*P*<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between women and men with T1DM. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

III. Modulation of the proteomic composition of L-HDL-P in women vs. men with obesity

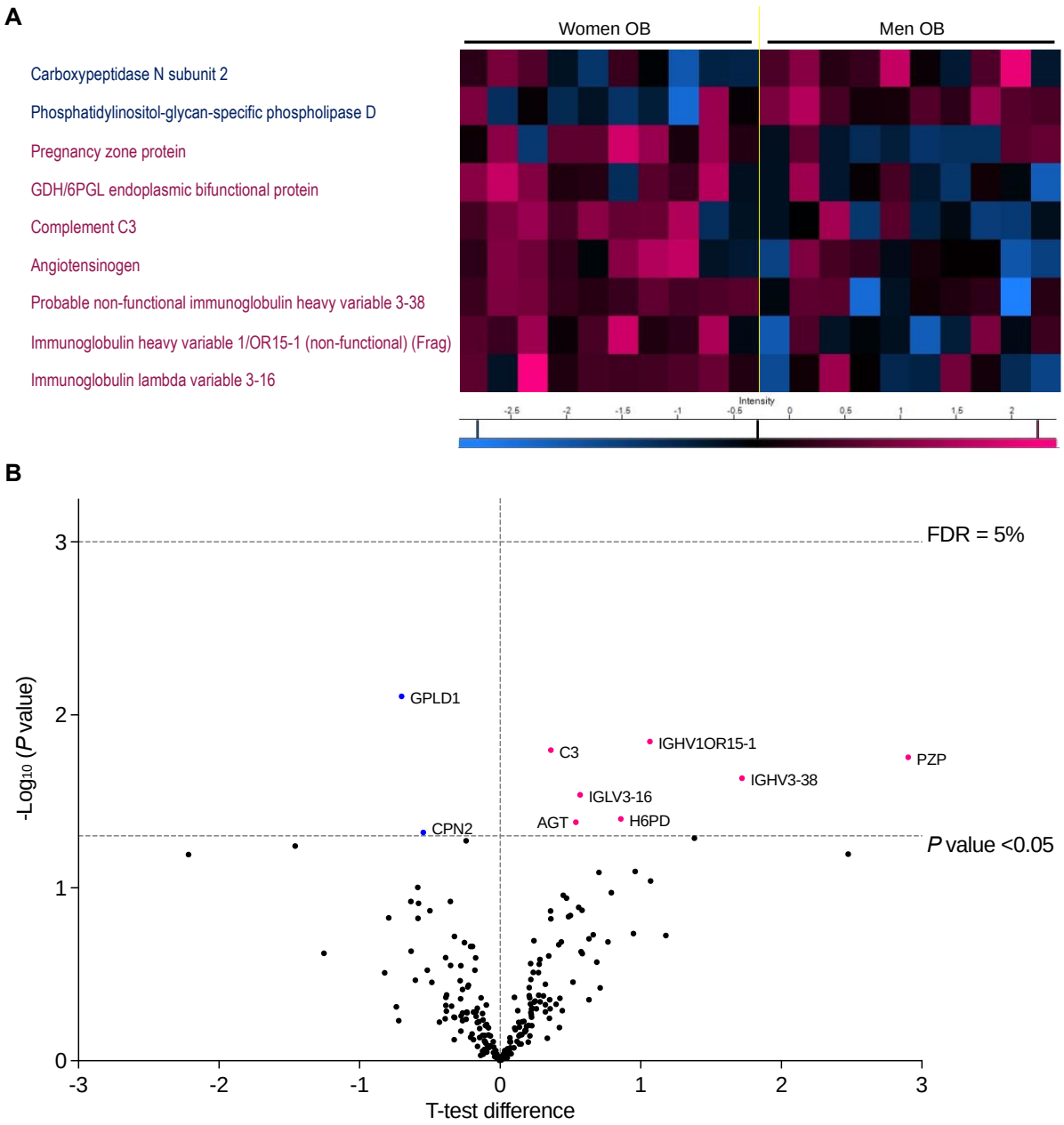

Figure 5.11 *Significantly changed proteins on large HDL-P between women and men with obesity*

HDL was isolated via FPLC from women and men with obesity (*n*=10 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between men and women were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (*P*<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between women and men with obesity. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

IV. Modulation of the proteomic composition of L-HDL-P in women vs. men with T2DM

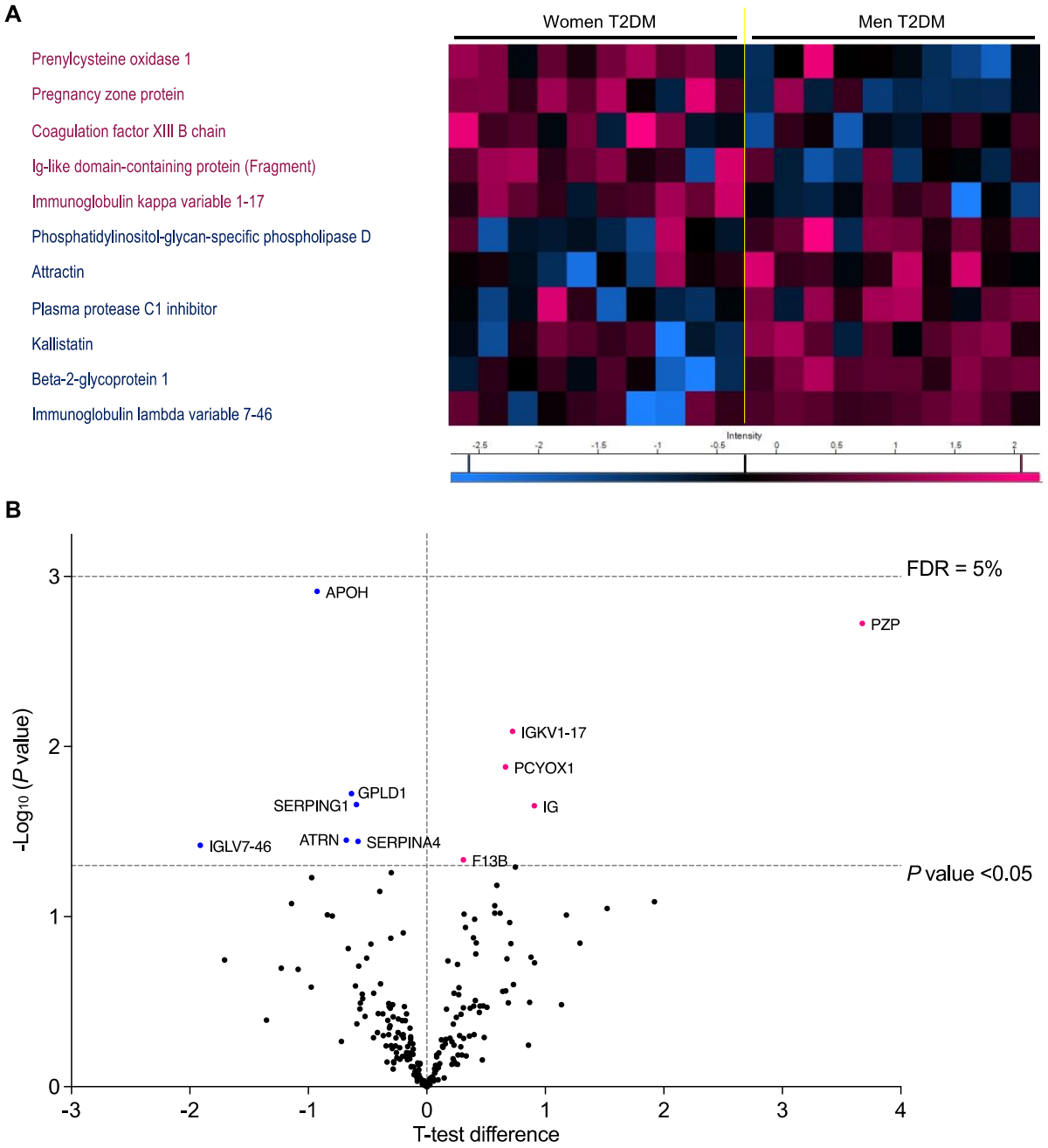

Figure 5.12 *Significantly changed proteins on large HDL-P between women and men with T2DM.*
HDL was isolated via FPLC from women and men with T2DM (n=10 per group). Differences in HDL protein levels on large HDL-P (fractions 36-38) between men and women were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (P<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between women and men with T2DM. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a P value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

Appendix 5E – Modulation of the proteomic composition of S-HDL-P

I. Modulation of the proteomic composition of S-HDL-P in women vs. men without obesity

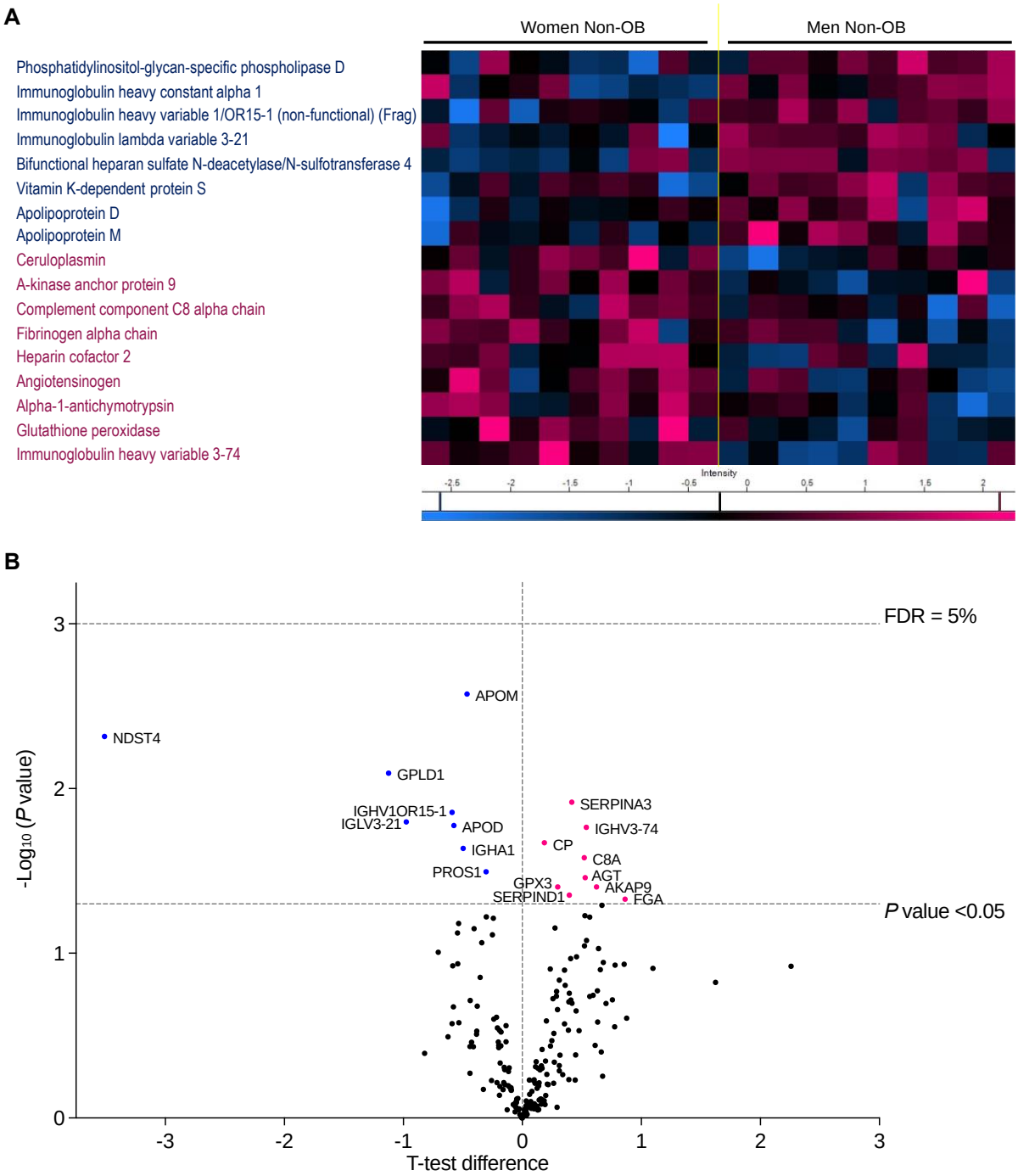

Figure 5.13 *Significantly changed proteins on small HDL-P between women and men without obesity.*

HDL was isolated via FPLC from women and men without obesity (n=10 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between men and women were identified by mass spectrometry.
(A) Perseus software was used to determine significantly (P<0.05) changed proteins and presented in a heatmap.
(B) Volcano plot showing the differences in protein abundance detected between women and men without obesity. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a P value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

II. Modulation of the proteomic composition of S-HDL-P in women vs. men with T1DM

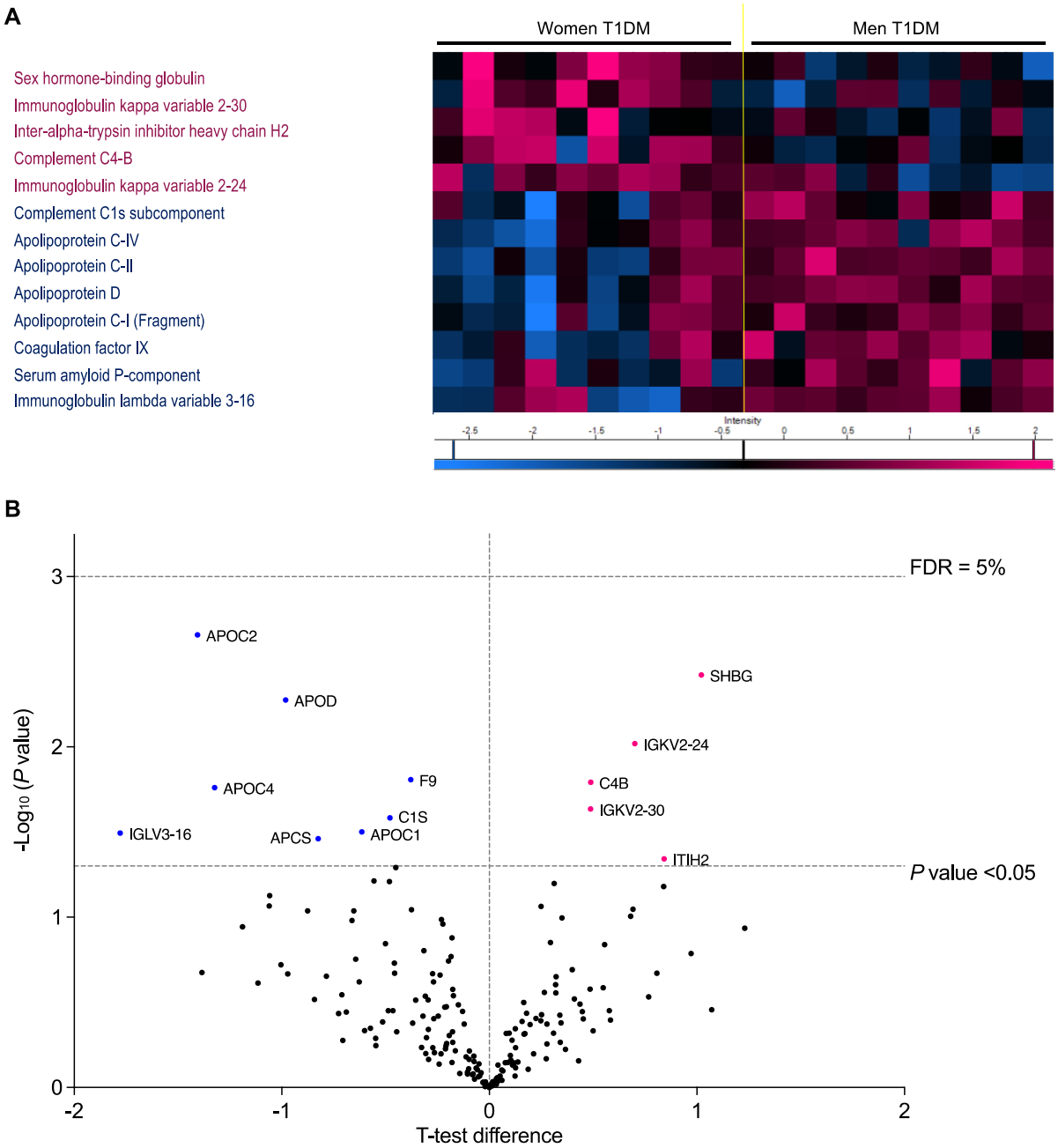

Figure 5.14 *Significantly changed proteins on small HDL-P between women and men with T1DM.*
HDL was isolated via FPLC from women and men with T1DM (*n*=10 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between men and women were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (*P*<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between women and men with T1DM. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

III. Modulation of the proteomic composition of S-HDL-P in women vs. men with obesity

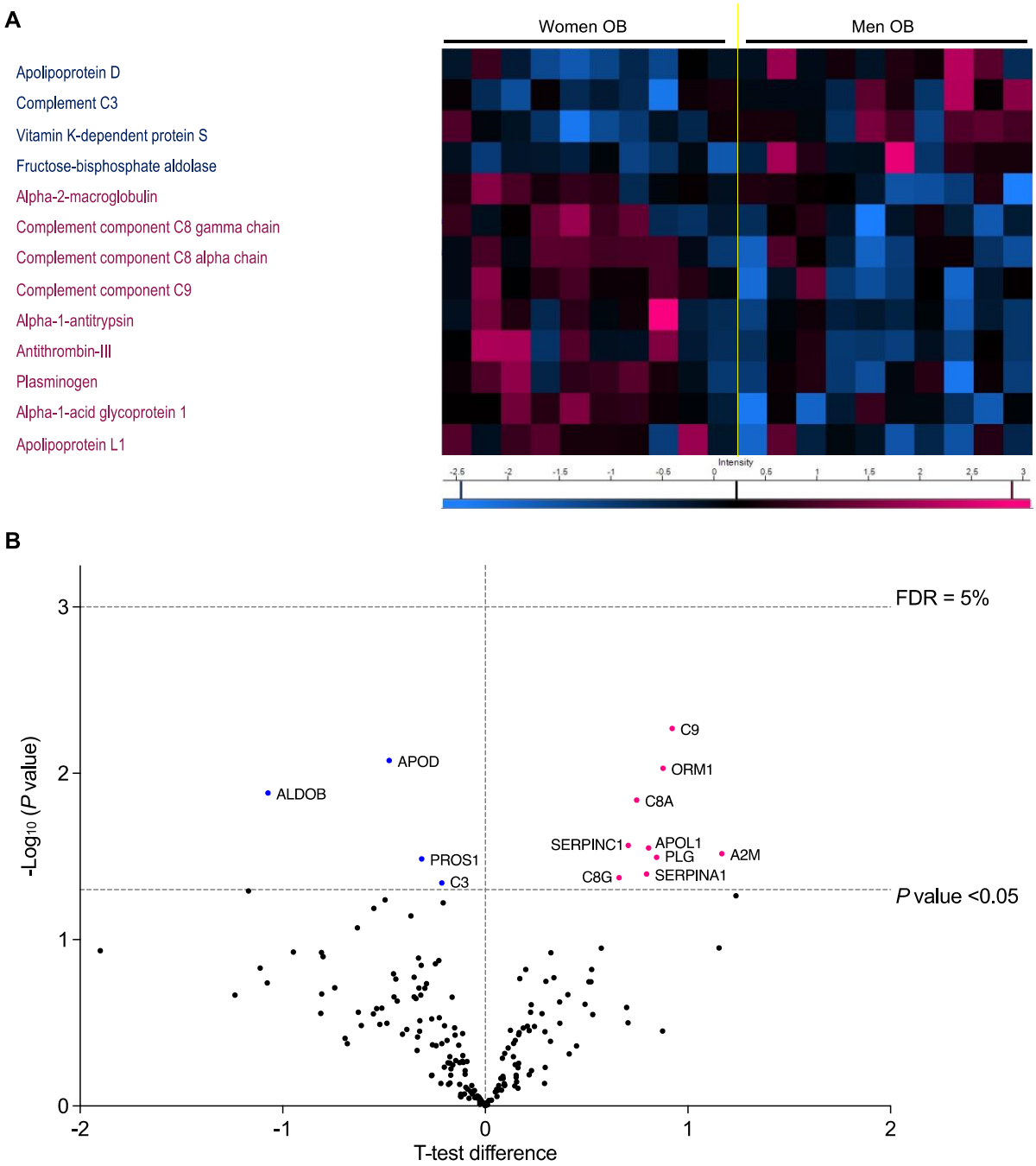

Figure 5.15 *Significantly changed proteins on small HDL-P between women and men with obesity.*
HDL was isolated via FPLC from women and men with obesity (*n*=10 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between men and women were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (*P*<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between women and men with obesity. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

IV. Modulation of the proteomic composition of S-HDL-P in women vs. men with T2DM

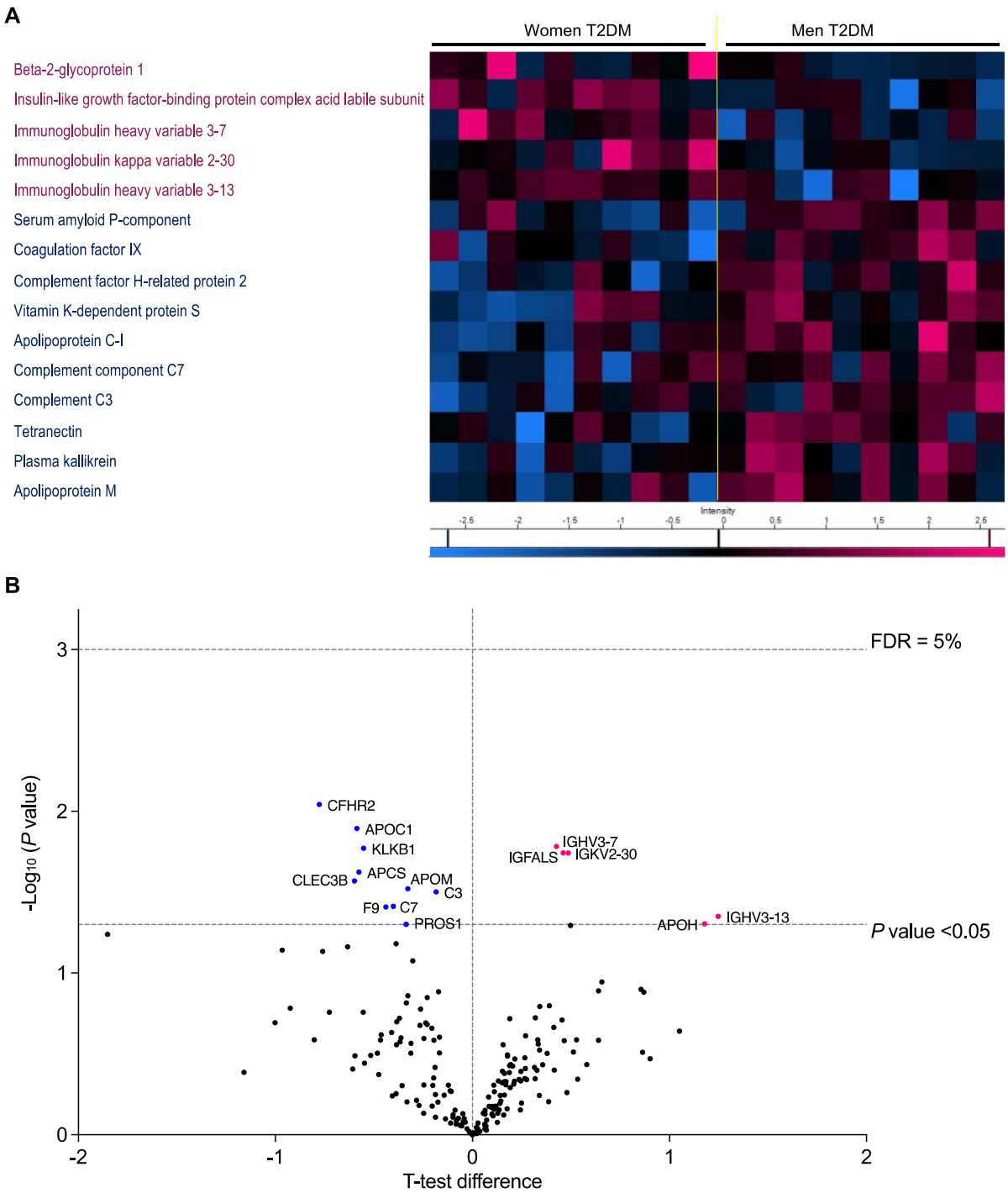

Figure 5.16 *Significantly changed proteins on small HDL-P between women and men with T2DM.*

HDL was isolated via FPLC from women and men with T2DM (*n*=10 per group). Differences in HDL protein levels on small HDL-P (fractions 40-42) between men and women were identified by mass spectrometry. **(A)** Perseus software was used to determine significantly (*P*<0.05) changed proteins and presented in a heatmap. **(B)** Volcano plot showing the differences in protein abundance detected between women and men with T2DM. Horizontal dotted lines indicate statistical thresholds for a false discovery rate (FDR) of 5% or a *P* value <0.05 calculated using a two-tailed t-test without adjustment for multiple comparison. Pink is up and blue is down in women w.r.t men.

Appendix 5F – Correlations between HDL-associated proteins on L-HDL-P and HDL-CEC parameters in women and men across the groups

Table 5.13 *Proteins on large HDL that significantly correlated with total efflux in women and men.*

L-HDL-associated protein	All		Non-OB		T1DM		OB		T2DM	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
Adipocyte plasma membrane-associated protein	0.181	0.426**	0.518	0.225	0.562	-0.184	0.110	0.751*	−0.663*	0.557
Alpha-1-antitrypsin	0.176	0.127	-0.367	0.493	0.345	-0.224	0.419	0.471	0.661*	-0.430
Alpha-2-HS-glycoprotein	0.293	0.354*	0.287	0.364	-0.074	-0.055	0.244	0.449	0.535	0.544
Antithrombin-III	0.017	0.065	-0.308	0.555	−0.679*	-0.374	0.432	-0.128	0.078	0.032
Apolipoprotein A-I	0.315	0.088	0.057	0.413	-0.377	0.085	0.646*	0.118	-0.351	0.009
Apolipoprotein A-II	0.574***	0.328*	0.473	0.693*	0.205	0.036	0.813**	0.504	-0.117	0.416
Apolipoprotein B-100	0.170	0.133	0.660*	0.424	0.253	0.364	0.018	-0.174	0.246	0.221
Apolipoprotein C-I	0.426**	0.250	0.363	0.482	-0.145	0.340	0.653*	0.247	0.339	0.506
Apolipoprotein C-II	0.197	0.245	0.640*	0.316	0.109	0.160	-0.026	-0.133	0.468	0.571
Apolipoprotein C-III	0.406*	0.257	0.420	0.236	0.339	0.211	0.690*	0.132	0.040	0.473
Apolipoprotein C-IV	0.296	-0.136	0.523	−0.720*	-0.103	0.015	0.667	-0.039	0.486	0.431
Apolipoprotein D	0.518***	0.021	0.507	0.119	0.142	0.081	0.718*	-0.514	-0.255	0.522
Apolipoprotein M	0.288	0.337*	0.352	0.716*	0.003	0.335	0.384	0.347	-0.068	0.616
Beta-2-glycoprotein 1	0.285	0.130	-0.012	0.329	0.324	-0.189	0.190	-0.036	-0.250	0.688*
Beta-Ala-His dipeptidase	-0.076	0.188	0.039	-0.181	0.271	0.657*	-0.050	0.094	-0.193	0.115
Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 4	0.095	−0.349*	0.391	-0.364	0.057	-0.419	0.075	-0.175	0.308	-0.293
C4b-binding protein alpha chain	0.111	0.399*	-0.261	0.355	0.438	0.777**	0.029	0.343	0.141	0.012
Cadherin-13	0.424**	-0.078	0.680*	0.084	0.017	-0.024	0.565	-0.125	0.164	0.257
Cadherin-5	0.040	0.328*	−0.822**	0.731*	-0.142	0.902***	0.196	-0.587	0.177	0.017
Coagulation factor XIII B chain	0.113	0.314*	0.370	0.315	0.311	0.329	0.278	0.539	-0.275	0.436
Complement C4-A	0.063	-0.209	-0.468	-0.132	0.493	-0.220	0.035	-0.173	0.396	−0.693*
Complement C4-B	−0.363*	-0.079	-0.545	-0.568	0.189	-0.069	-0.377	-0.033	0.300	-0.120
Complement C5	-0.287	0.254	-0.296	0.070	0.254	0.685*	-0.105	0.097	-0.528	0.276
Complement component C6	−0.318*	0.137	-0.547	0.434	-0.229	0.295	-0.616	0.437	0.229	-0.573
Complement component C7	0.060	0.369*	-0.388	0.504	0.026	0.112	0.366	0.245	-0.130	-0.027
Complement component C8 alpha chain	0.414*	0.087	0.663	0.166	0.284	0.196	0.530	-0.405	0.167	0.329
Complement component C8 gamma chain	-0.089	0.113	-0.290	-0.405	0.227	−0.738*	-0.155	0.624	0.009	-0.155
Complement factor H	-0.237	-0.245	-0.076	-0.262	0.103	-0.379	-0.102	-0.254	−0.794**	-0.343
Complement factor H-related protein 1	0.339*	0.179	0.487	0.141	0.297	0.313	0.259	-0.286	0.111	0.362
Complement subcomponent C1r	-0.296	-0.256	-0.342	-0.230	-0.279	-0.342	0.126	-0.238	−0.815**	-0.096
Endoplasmic reticulum chaperone BiP	0.200	0.120	0.736*	0.283	0.065	0.513	-0.024	-0.340	0.442	0.402
Extracellular matrix protein 1	-0.151	0.166	-0.246	0.477	-0.293	0.450	0.188	0.148	−0.650*	0.091
Fibronectin	0.007	0.080	-0.133	0.296	0.172	-0.094	-0.137	0.288	−0.828**	-0.043
Fibulin-1	-0.010	−0.384*	-0.128	−0.792*	-0.090	-0.524	-0.158	0.094	0.273	−0.739*
Ficolin-3	-0.365	0.060	-0.590	-0.197	0.138	-0.316	-0.584	0.786*	0.541	-0.554
Gelsolin	0.412**	-0.119	0.295	0.176	0.112	-0.108	0.628	-0.193	-0.168	0.265
Haptoglobin-related protein	-0.180	-0.104	-0.588	0.013	0.397	-0.259	−0.673*	-0.208	-0.373	-0.067
Hemopexin	-0.002	0.335*	0.396	0.202	-0.340	0.317	-0.295	0.328	0.318	0.659*
Histidine-rich glycoprotein	0.034	-0.006	-0.453	-0.389	-0.084	-0.342	0.262	0.679*	0.197	0.117
Hyaluronan-binding protein 2	0.520**	-0.232	0.837**	-0.134	0.352	-0.322	0.421	-0.495	0.625	0.357
Ig-like domain-containing protein (Fragment)	0.350*	-0.045	0.454	-0.654	0.052	0.124	0.522	-0.632	0.595	0.243
Ig-like domain-containing protein (Fragment)	0.168	−0.426**	0.602	-0.606	-0.078	-0.299	0.795**	−0.639*	-0.173	-0.342
Immunoglobulin heavy constant alpha 2 (Fragment)	0.433*	0.120	0.498	0.220	-0.046	0.345	0.421	0.235	0.560	-0.719
Immunoglobulin heavy constant gamma 3	0.149	−0.578****	0.211	−0.795**	0.156	-0.495	0.193	-0.602	0.015	-0.536
Immunoglobulin heavy constant gamma 4	0.242	-0.206	-0.176	-0.350	0.202	0.133	0.336	-0.068	0.662*	-0.626
Immunoglobulin heavy variable 1-18	0.052	-0.298	0.601	-0.364	−0.679*	-0.378	0.396	-0.535	0.124	-0.303
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	0.130	0.037	0.115	-0.380	-0.168	0.730*	0.219	-0.345	0.092	0.344
Immunoglobulin heavy variable 3-49	0.127	-0.146	-0.215	0.105	−0.739*	0.244	0.507	-0.416	0.419	-0.260
Immunoglobulin heavy variable 3-7	0.187	-0.280	0.480	-0.186	-0.434	0.057	0.554	−0.672*	0.078	-0.295
Immunoglobulin heavy variable 3-72	0.135	−0.317*	0.709*	-0.264	-0.287	0.138	0.067	−0.671*	0.279	-0.015
Immunoglobulin heavy variable 3-73	0.236	-0.054	0.295	-0.186	-0.299	0.473	0.734*	-0.288	-0.601	0.233
Immunoglobulin heavy variable 4-28	0.106	-0.106	0.147	-0.246	−0.727*	-0.108	0.671*	-0.504	0.613	0.292
Immunoglobulin heavy variable 4-38-2	0.204	-0.156	0.415	-0.124	-0.391	0.118	0.692*	-0.455	0.091	-0.592
Immunoglobulin heavy variable 6-1	0.300	0.145	0.404	−0.790**	−0.715*	0.405	0.789**	0.007	0.697*	0.362
Immunoglobulin kappa variable 1-33	0.224	−0.334*	0.111	-0.291	-0.198	0.009	0.429	-0.272	0.377	−0.705*
Immunoglobulin kappa variable 2-30	-0.132	0.029	0.032	-0.186	-0.342	0.780*	-0.284	-0.502	0.235	−0.802**
Immunoglobulin kappa variable 2D-29	0.351*	−0.347*	0.430	-0.525	0.407	-0.104	0.331	-0.220	0.492	-0.591
Immunoglobulin lambda constant 3	0.017	−0.398*	0.122	-0.557	-0.274	-0.151	-0.055	-0.474	0.136	-0.332
Immunoglobulin lambda constant 7 (Fragment)	-0.125	-0.138	-0.445	-0.414	−0.811**	0.010	0.398	0.081	-0.351	−0.723*
Immunoglobulin lambda variable 1-51	-0.052	-0.001	0.698*	-0.357	-0.063	-0.258	-0.085	0.027	0.123	0.416
Immunoglobulin lambda variable 2-11	0.025	-0.033	-0.308	−0.670*	-0.338	0.441	0.357	0.020	0.046	-0.326
Immunoglobulin lambda variable 3-10	0.122	−0.400*	0.472	-0.258	0.348	0.024	0.415	-0.496	0.112	-0.223
Immunoglobulin lambda variable 3-21	-0.263	−0.458**	-0.519	-0.262	-0.355	-0.418	-0.040	-0.376	-0.344	-0.649
Immunoglobulin lambda variable 3-9	0.044	−0.372*	0.105	-0.087	-0.576	0.137	0.485	-0.603	-0.019	-0.280
Immunoglobulin lambda variable 5-45 (Fragment)	0.008	−0.322*	-0.019	-0.399	−0.927***	-0.037	0.238	-0.287	0.505	-0.153
Immunoglobulin lambda variable 8-61	0.448**	0.140	0.127	-0.268	0.274	0.521	0.760*	-0.287	0.281	0.148
Inter-alpha-trypsin inhibitor heavy chain H1	0.264	-0.057	0.826**	-0.320	0.216	-0.054	-0.042	0.055	0.165	0.030
ITIH4 protein	0.322*	0.337*	0.637*	0.478	-0.169	0.613	0.646*	0.234	0.295	0.490
Keratin, type I cytoskeletal 9	-0.028	-0.257	-0.047	0.133	-0.074	-0.470	0.272	-0.508	−0.890**	-0.680
L-selectin	0.027	-0.211	-0.070	-0.091	-0.296	-0.108	0.003	-0.466	−0.731*	-0.025
Macrophage colony-stimulating factor 1 receptor	-0.111	0.234	0.137	-0.364	−0.818*	0.773*	-0.045	0.105	-0.417	0.025
Mast/stem cell growth factor receptor Kit	0.241	-0.087	0.069	-0.240	-0.097	-0.010	0.594	-0.296	−0.804**	0.303
Phosphatidylcholine-sterol acyltransferase	0.151	0.405**	0.568	0.231	0.090	0.216	0.542	0.510	-0.619	0.407
Phospholipid transfer protein	0.160	-0.136	0.149	-0.230	-0.195	0.734*	0.472	-0.377	-0.240	0.521
Plexin domain-containing protein 2	0.045	0.165	0.824**	-0.212	-0.366	0.255	0.345	0.157	0.293	0.398
Properdin	-0.081	-0.027	0.102	−0.633*	-0.109	-0.395	-0.059	0.145	0.226	0.304
SAA2-SAA4	0.409**	0.324*	-0.188	0.473	0.729*	0.115	0.420	0.621	0.415	0.001
Scavenger receptor cysteine-rich type 1 protein M130	-0.072	0.059	0.633*	−0.668*	-0.104	-0.519	-0.088	0.225	0.144	0.682*
Serum amyloid P-component	-0.217	0.261	0.008	0.589	0.218	0.564	-0.338	-0.115	−0.760*	0.557
Sulfhydryl oxidase 1	-0.276	0.151	-0.323	-0.160	0.016	-0.062	-0.173	-0.179	−0.679*	0.319
Tetranectin	0.260	-0.228	0.559	0.642	-0.651	0.102	0.594	−0.708*	-0.042	0.210
Transthyretin	0.301	0.005	0.662*	0.421	-0.057	-0.461	0.271	-0.143	0.064	-0.072
Vasorin	-0.183	-0.268	0.085	-0.356	-0.290	−0.675*	-0.279	-0.253	-0.085	0.009
Vitamin K-dependent protein S	0.054	0.271	0.049	0.289	0.714*	0.252	0.250	0.279	-0.229	0.221

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 5.14 *Proteins on large HDL that significantly correlated with ABCA1-dependent efflux in women and men.*

L-HDL-associated protein	All		Non-OB		T1DM		OB		T2DM	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
Adipocyte plasma membrane-associated protein	-0.124	0.250	0.294	0.020	-0.167	-0.252	-0.324	0.727*	-0.625	0.030
Alpha-1-antichymotrypsin	0.074	0.287	0.195	0.635*	0.061	0.586	-0.147	-0.219	-0.111	0.564
Alpha-1-antitrypsin	0.180	0.215	-0.321	0.728*	0.459	-0.038	0.421	0.274	0.705*	-0.416
Alpha-1B-glycoprotein	0.170	0.008	0.331	−0.657*	0.136	0.235	0.231	0.118	-0.009	0.294
Alpha-2-antiplasmin	0.118	0.081	-0.253	0.633*	0.505	-0.419	-0.079	-0.351	0.534	0.094
Alpha-2-HS-glycoprotein	0.231	0.355*	-0.033	0.566	-0.098	0.059	0.316	0.214	0.487	0.414
Angiotensinogen	-0.029	0.121	-0.307	0.666*	-0.184	-0.119	0.236	-0.323	-0.248	0.012
Antithrombin-III	0.121	0.209	-0.289	0.746*	-0.021	-0.232	0.524	0.035	0.556	0.431
Apolipoprotein A-II	0.332*	0.270	-0.001	0.635*	0.118	-0.221	0.743*	0.551	-0.445	0.476
Apolipoprotein B-100	0.091	0.029	0.668*	0.539	0.092	0.210	-0.330	-0.310	0.260	-0.156
Apolipoprotein C-I	0.121	0.200	-0.210	0.519	-0.538	0.237	0.693*	0.036	0.041	0.624
Apolipoprotein C-III	0.264	0.310	0.257	0.016	0.045	0.375	0.638	0.241	-0.402	0.736*
Apolipoprotein D	0.252	-0.156	0.076	-0.181	-0.431	0.056	0.820**	-0.430	-0.465	0.132
Apolipoprotein E	-0.209	0.395*	-0.480	0.052	-0.462	-0.294	0.242	0.533	-0.406	0.712*
Apolipoprotein M	-0.046	0.122	-0.072	0.548	−0.795*	-0.031	0.308	0.191	-0.375	0.408
Beta-Ala-His dipeptidase	-0.144	0.122	-0.023	-0.504	-0.233	0.674*	0.127	0.210	-0.385	0.101
C4b-binding protein alpha chain	-0.167	0.283	-0.093	0.391	-0.331	0.670*	-0.375	0.114	-0.134	-0.034
Cadherin-13	0.357*	-0.145	0.594	-0.099	-0.387	0.408	0.551	-0.334	-0.096	0.154
Cadherin-5	-0.144	0.200	−0.695*	0.847**	−0.805**	0.814**	0.134	−0.685*	-0.065	-0.463
Carboxypeptidase N subunit 2	0.048	-0.119	0.234	0.018	0.719*	-0.265	0.013	0.010	-0.605	0.236
Cartilage oligomeric matrix protein	0.069	-0.139	0.126	-0.405	-0.341	-0.070	0.180	-0.491	0.670*	0.446
Ceruloplasmin	0.246	0.359*	0.478	0.732*	0.322	0.218	-0.054	0.563	0.229	0.625
Complement C1r subcomponent-like protein	0.375*	-0.012	-0.017	-0.175	0.504	-0.004	0.470	-0.093	0.033	0.035
Complement C1s subcomponent	−0.445**	-0.001	-0.336	-0.408	−0.823**	0.127	−0.702*	0.507	0.069	-0.318
Complement C4-A	-0.084	-0.261	-0.334	-0.197	-0.254	-0.155	-0.235	-0.071	0.327	−0.756*
Complement C4-B	−0.359*	-0.028	-0.331	-0.592	-0.654	0.018	-0.459	0.150	0.399	-0.223
Complement component C6	-0.287	0.075	-0.382	0.398	-0.065	-0.127	−0.730*	0.221	0.008	-0.159
Complement component C9	0.046	0.051	0.240	0.638*	-0.057	0.046	0.141	-0.456	0.178	-0.134
Extracellular matrix protein 1	-0.086	0.073	-0.176	0.055	0.267	0.470	0.115	0.149	−0.869**	-0.188
Fibulin-1	0.039	−0.389*	-0.051	-0.567	-0.166	−0.637*	0.231	-0.181	0.297	-0.357
Ficolin-3	-0.232	0.088	-0.068	-0.356	-0.501	-0.647	-0.456	0.887**	-0.545	-0.629
Gelsolin	0.445**	-0.195	0.194	-0.046	0.456	-0.060	0.774*	-0.387	-0.143	0.107
Haptoglobin	-0.042	0.025	-0.292	0.381	0.464	-0.315	-0.163	0.370	−0.811**	-0.396
Haptoglobin-related protein	-0.150	-0.055	-0.338	0.101	-0.245	-0.312	−0.725*	0.060	-0.384	-0.238
Hemoglobin subunit alpha	-0.051	-0.023	-0.563	0.323	0.408	−0.637*	-0.468	-0.202	−0.684*	0.216
Hemoglobin subunit beta	-0.101	0.103	-0.591	0.306	0.426	−0.655*	-0.474	-0.137	−0.669*	0.282
Hemopexin	-0.022	0.380*	0.210	0.180	-0.053	0.483	-0.075	0.109	0.377	0.645*
Histidine-rich glycoprotein	0.116	0.030	−0.817**	-0.184	0.565	-0.414	0.230	0.767**	0.419	-0.197
Hyaluronan-binding protein 2	0.525**	-0.159	0.710*	-0.078	0.687	-0.461	0.522	-0.414	-0.142	0.164
Ig-like domain-containing protein (Fragment)	0.307	-0.294	0.449	-0.618	0.567	0.112	0.680*	-0.482	-0.060	-0.133
Immunoglobulin heavy constant alpha 2 (Fragment)	0.219	0.044	0.239	0.232	−0.756*	0.590	0.533	0.140	0.408	-0.694
Immunoglobulin heavy constant gamma 3	0.031	−0.496**	-0.150	−0.677*	0.425	-0.189	0.143	-0.441	0.001	-0.492
Immunoglobulin heavy variable 1-45	0.070	-0.325	0.333	-0.662	0.000	0.379	-0.321	-0.105	-0.338	−0.770*
Immunoglobulin heavy variable 1-69	0.197	-0.034	0.076	-0.080	0.457	0.751*	0.284	-0.475	-0.253	-0.297
Immunoglobulin heavy variable 1/OR15-1 (non-functional) (Fragment)	0.028	-0.116	0.254	-0.523	-0.072	0.806*	-0.072	-0.498	0.574	0.025
Immunoglobulin heavy variable 3-15	0.112	-0.170	-0.186	-0.289	0.637	0.710*	0.043	−0.701*	0.062	0.250
Immunoglobulin heavy variable 3-7	0.342*	-0.213	0.475	-0.384	0.299	0.475	0.559	-0.605	0.090	-0.072
Immunoglobulin heavy variable 3-72	0.117	-0.229	0.531	-0.461	0.160	0.503	-0.099	−0.801**	-0.230	0.261
Immunoglobulin heavy variable 3-73	0.343*	-0.132	0.412	-0.436	0.203	0.664	0.810**	-0.474	-0.292	-0.004
Immunoglobulin heavy variable 4-28	0.188	-0.055	0.197	-0.315	-0.103	0.284	0.745*	−0.661*	0.516	0.449
Immunoglobulin heavy variable 4-38-2	0.329*	-0.237	0.509	-0.363	0.429	0.406	0.718*	-0.552	-0.285	-0.549
Immunoglobulin heavy variable 6-1	0.208	0.237	0.256	-0.600	-0.305	0.347	0.684*	-0.156	0.415	0.522
Immunoglobulin kappa variable 1-33	0.332*	-0.299	0.252	-0.469	0.455	0.340	0.377	-0.171	0.089	-0.510
Immunoglobulin kappa variable 1-8	0.282	0.018	0.499	0.071	-0.023	0.651*	0.465	-0.216	0.198	-0.255
Immunoglobulin kappa variable 2-30	-0.257	-0.029	0.108	-0.402	-0.247	0.739*	-0.435	-0.512	0.252	-0.585
Immunoglobulin kappa variable 2D-29	0.173	−0.365*	-0.101	-0.652	0.373	-0.169	0.233	-0.347	-0.185	-0.425
Immunoglobulin kappa variable 6-21	0.121	−0.322*	-0.126	-0.159	0.565	-0.046	0.292	-0.575	0.364	-0.461
Immunoglobulin lambda constant 7 (Fragment)	-0.187	-0.195	-0.586	-0.292	−0.670*	-0.093	0.522	-0.165	0.149	-0.305
Immunoglobulin lambda variable 1-47	0.374*	-0.292	0.234	-0.195	0.507	-0.111	0.761*	-0.415	0.461	-0.244
Immunoglobulin lambda variable 3-10	0.183	−0.428**	0.556	-0.476	0.204	0.301	0.467	-0.593	0.260	-0.241
Immunoglobulin lambda variable 3-21	-0.334	−0.396*	-0.517	-0.113	-0.786	-0.181	-0.003	-0.451	0.764	-0.663
Immunoglobulin lambda variable 3-9	0.098	−0.331*	0.080	-0.275	-0.007	0.480	0.357	−0.652*	0.130	-0.070
Immunoglobulin lambda variable 8-61	0.465**	-0.020	0.054	-0.480	0.689	0.317	0.822**	-0.354	0.200	-0.064
Immunoglobulin lambda variable 9-49	0.085	-0.121	-0.210	-0.222	0.348	0.836*	-0.167	-0.300	-0.032	-0.387
Interleukin-1 receptor accessory protein	0.360*	0.066	0.282	0.123	0.473	-0.281	0.228	0.547	0.209	-0.061
ITIH4 protein	0.413**	0.297	0.754*	0.621	0.228	0.319	0.457	0.182	0.197	0.237
Kininogen-1	0.229	-0.002	0.538	0.507	-0.005	−0.670*	0.272	-0.201	0.575	-0.072
Low affinity immunoglobulin gamma Fc region receptor III-A	-0.061	0.220	-0.262	-0.415	0.511	-0.414	-0.081	0.389	0.235	0.796**
Lumican	0.126	-0.070	-0.191	0.020	0.083	0.345	0.517	-0.414	0.471	0.784**
Mannan-binding lectin serine protease 1	0.051	0.098	0.528	-0.484	-0.271	-0.009	-0.175	0.279	0.676*	0.167
Mast/stem cell growth factor receptor Kit	0.181	-0.059	-0.266	-0.332	0.125	0.126	0.725*	-0.338	-0.476	0.328
Phosphatidylcholine-sterol acyltransferase	-0.103	0.359*	0.443	0.100	-0.548	0.456	0.335	0.484	-0.259	0.169
Phosphatidylinositol-glycan-specific phospholipase D	-0.147	-0.035	0.163	-0.515	-0.074	-0.012	-0.331	-0.443	0.687*	0.211
Plasminogen	0.229	0.117	0.035	0.747*	0.228	-0.151	0.453	-0.320	-0.288	-0.083
Plexin domain-containing protein 2	0.028	0.021	0.692*	-0.442	-0.568	0.057	0.387	-0.046	0.489	0.119
Properdin	-0.117	-0.105	0.056	−0.684*	-0.393	-0.442	-0.164	-0.021	0.216	-0.088
Protein AMBP	0.138	-0.292	0.397	−0.649*	-0.358	0.121	0.303	-0.421	0.344	-0.074
Serotransferrin	0.265	0.134	-0.118	0.653*	0.321	0.042	0.640	-0.479	0.099	0.506
Serum amyloid P-component	-0.118	0.197	0.121	0.692*	0.457	0.523	0.105	-0.197	-0.491	0.093
Serum paraoxonase/lactonase 3	0.070	-0.166	0.122	-0.270	−0.700*	-0.266	-0.489	-0.058	-0.436	0.248
Transthyretin	0.364*	0.186	0.505	0.679*	0.258	-0.356	0.316	-0.013	0.024	0.102
Vasorin	-0.026	−0.347*	-0.127	-0.262	0.280	-0.411	-0.390	-0.312	0.370	-0.492

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 5.15 *Proteins on large HDL that significantly correlated with ABCA1-independent efflux in women and men.*

L-HDL-associated protein	All		Non-OB		T1DM		OB		T2DM	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
A-kinase anchor protein 9	-0.025	-0.304	-0.061	−0.708*	-0.971	-0.789	0.104	-0.101	-0.480	0.455
Adipocyte plasma membrane-associated protein	0.469**	0.470**	0.296	0.413	0.689*	-0.043	0.453	0.559	0.011	0.675*
Alpha-2-HS-glycoprotein	0.142	0.209	0.438	-0.017	0.035	-0.171	-0.048	0.644*	0.077	0.198
Alpha-2-macroglobulin	0.008	0.309	-0.031	-0.399	-0.032	0.009	0.201	0.205	-0.393	0.656*
Apolipoprotein A-I	0.432**	0.228	0.646*	0.448	0.138	0.079	0.580	-0.008	-0.284	0.272
Apolipoprotein A-II	0.450**	0.265	0.647*	0.532	0.066	0.323	0.475	0.289	0.311	-0.040
Apolipoprotein A-IV	0.116	0.099	0.654*	0.343	0.281	0.277	-0.497	0.364	0.031	-0.146
Apolipoprotein C-I	0.513***	0.211	0.788**	0.273	0.428	0.345	0.134	0.470	0.565	-0.104
Apolipoprotein C-II	0.150	0.323*	0.316	0.487	0.254	0.182	-0.177	0.216	0.812**	0.364
Apolipoprotein C-IV	0.482**	-0.008	0.747*	-0.598	0.642	-0.042	0.344	0.010	-0.556	0.716
Apolipoprotein D	0.474**	0.230	0.587	0.461	0.578	0.083	0.144	-0.477	0.207	0.509
Apolipoprotein E	0.322*	-0.022	0.809**	0.658*	0.214	-0.037	-0.257	0.391	0.330	-0.229
Apolipoprotein F	0.349*	0.254	0.324	0.648*	0.522	-0.213	0.052	-0.249	-0.010	0.441
Apolipoprotein M	0.530***	0.465**	0.581	0.688*	0.830**	0.647*	0.182	0.461	0.440	0.296
C4b-binding protein alpha chain	0.417**	0.380*	-0.226	0.189	0.746*	0.640*	0.284	0.562	0.778**	0.056
Cadherin-5	0.271	0.354*	-0.151	0.336	0.707*	0.700*	0.012	-0.273	0.506	0.578
Carboxypeptidase N subunit 2	-0.245	-0.110	-0.258	0.572	−0.714*	−0.678*	0.293	0.184	-0.347	-0.275
Ceruloplasmin	-0.114	-0.039	−0.705*	-0.060	-0.405	-0.203	0.440	0.075	-0.535	0.030
Coagulation factor XII	−0.423*	0.005	-0.535	0.074	-0.671	0.517	-0.581	-0.532	-0.044	0.795*
Complement C1q subcomponent subunit B (Fragment)	0.050	0.305	-0.090	0.504	-0.070	0.445	0.205	0.309	0.784*	0.231
Complement C1q subcomponent subunit C	0.139	0.232	-0.235	-0.090	0.403	0.375	0.299	0.402	0.965***	0.118
Complement C1s subcomponent	0.153	0.121	-0.027	-0.208	0.689*	0.292	0.071	0.242	0.092	-0.129
Complement C3	-0.292	-0.020	−0.825**	-0.134	-0.108	0.524	0.353	0.071	-0.036	-0.250
Complement C4-A	0.222	-0.060	-0.172	-0.004	0.716*	-0.222	0.136	-0.265	-0.185	0.023
Complement C4-B	-0.071	-0.110	-0.281	-0.346	0.854**	-0.147	-0.226	-0.280	0.084	0.114
Complement component C6	-0.102	0.158	-0.213	0.334	-0.143	0.688*	-0.087	0.609	0.562	-0.540
Complement component C7	0.060	0.435**	-0.245	0.538	0.311	0.471	0.116	0.292	-0.097	0.055
Complement component C8 gamma chain	0.090	0.062	0.296	-0.329	-0.126	−0.785*	0.011	0.723	0.164	-0.071
Complement factor H-related protein 1	0.177	0.328*	0.213	0.512	0.181	0.491	-0.163	0.137	-0.118	0.498
Extracellular matrix protein 1	-0.121	0.213	-0.090	0.840**	-0.546	0.274	0.445	0.101	0.098	0.342
Fibronectin	0.044	0.161	-0.519	0.337	0.111	0.107	0.100	0.050	-0.381	0.638*
Fibulin-1	-0.061	-0.216	-0.106	−0.818**	0.083	-0.215	-0.453	0.420	0.160	-0.583
GDH/6PGL endoplasmic bifunctional protein	-0.138	0.013	-0.049	0.486	0.171	-0.509	-0.340	-0.118	0.039	0.642*
Hemoglobin subunit alpha	-0.140	-0.012	0.181	0.242	-0.092	-0.060	−0.660*	−0.777**	-0.005	-0.426
Hemoglobin subunit beta	-0.155	-0.061	0.034	0.271	-0.140	-0.038	−0.643*	−0.782**	0.050	-0.452
Hepatocyte growth factor activator	0.131	0.332*	0.082	0.117	0.443	0.363	-0.341	0.401	0.154	0.446
Ig-like domain-containing protein (Fragment)	-0.168	−0.416**	0.193	-0.386	-0.661	−0.675*	0.571	−0.667*	-0.399	-0.277
Immunoglobulin heavy constant alpha 1	-0.090	−0.324*	0.111	-0.476	-0.205	-0.600	0.015	-0.414	-0.313	-0.040
Immunoglobulin heavy constant alpha 2 (Fragment)	0.384*	0.170	0.313	0.128	0.675	-0.076	0.195	0.301	-0.262	0.094
Immunoglobulin heavy constant delta	-0.163	0.063	0.182	0.078	0.466	0.654*	−0.637*	0.002	-0.388	0.046
Immunoglobulin heavy constant gamma 3	0.196	−0.446**	0.497	−0.678*	-0.299	−0.684*	0.578	−0.648*	-0.178	-0.095
Immunoglobulin heavy variable 1-18	-0.184	-0.289	0.127	-0.182	-0.541	−0.646*	0.156	-0.418	-0.300	-0.163
Immunoglobulin heavy variable 1-69	-0.258	0.025	-0.321	0.257	−0.756*	-0.067	-0.043	-0.151	-0.458	-0.012
Immunoglobulin heavy variable 3-13	-0.102	-0.055	0.120	-0.042	-0.463	-0.241	0.304	0.768*	-0.059	-0.166
Immunoglobulin heavy variable 3-7	-0.189	-0.249	-0.009	0.129	−0.709*	-0.448	0.118	-0.564	-0.068	-0.291
Immunoglobulin heavy variable 3-74	-0.250	-0.109	-0.151	-0.098	−0.775*	-0.004	0.171	-0.167	0.093	-0.015
Immunoglobulin heavy variable 3-9	−0.329*	0.067	-0.294	-0.278	-0.428	-0.162	-0.061	-0.073	−0.838**	0.491
Immunoglobulin heavy variable 4-38-2	-0.145	0.012	-0.144	0.221	−0.804**	-0.258	0.180	-0.177	0.044	-0.037
Immunoglobulin heavy variable 6-1	0.185	-0.028	0.195	−0.765**	-0.338	0.336	0.593	0.233	0.487	-0.166
Immunoglobulin kappa variable 1-6	0.192	0.096	-0.074	0.283	0.739*	0.337	0.003	-0.116	-0.157	-0.309
Immunoglobulin kappa variable 2D-29	0.306	-0.187	0.728*	-0.193	-0.015	-0.001	0.127	0.036	0.837	-0.250
Immunoglobulin lambda constant 3	-0.082	−0.416**	0.241	-0.488	-0.500	-0.572	0.063	-0.518	-0.479	-0.258
Immunoglobulin lambda variable 1-51	-0.166	0.175	0.593	-0.192	−0.696*	-0.256	-0.296	0.231	0.069	0.629
Immunoglobulin lambda variable 3-21	0.054	−0.384*	0.035	-0.357	0.562	-0.551	-0.038	-0.218	0.796	-0.204
Immunoglobulin lambda variable 9-49	-0.264	-0.026	0.099	-0.221	−0.713*	0.452	-0.588	0.249	0.393	0.605
Insulin-like growth factor-binding protein 3	-0.023	0.409*	-0.058	0.764*	-0.082	0.468	0.476	0.186	0.314	0.384
Interleukin-1 receptor accessory protein	-0.082	0.357*	0.525	0.564	-0.405	-0.308	-0.087	0.349	-0.179	0.628
ITIH4 protein	-0.073	0.249	-0.185	0.134	-0.392	0.748*	0.591	0.239	0.140	0.342
Kininogen-1	-0.127	0.027	0.083	0.495	-0.165	-0.288	-0.208	-0.021	−0.676*	-0.024
Phosphatidylcholine-sterol acyltransferase	0.391*	0.297	0.156	0.323	0.653	-0.136	0.477	0.395	0.097	0.317
Pregnancy zone protein	0.230	0.110	-0.200	0.289	0.532	0.009	0.706*	0.547	0.146	0.153
Prenylcysteine oxidase 1	0.416**	0.438**	0.079	0.667*	0.710*	0.130	0.305	0.382	0.612	0.729*
SAA2-SAA4	0.570***	0.403**	0.327	0.121	0.800**	0.473	0.705*	0.432	0.382	0.215
Serum paraoxonase/arylesterase 1	0.253	-0.002	0.460	0.285	-0.134	−0.638*	0.725*	0.232	0.000	-0.252
Serum paraoxonase/lactonase 3	0.316	0.348*	0.378	0.450	0.718*	0.293	0.168	0.375	0.010	0.393
Tetranectin	0.095	-0.216	0.967	0.871	-0.443	0.015	-0.016	−0.743*	0.180	0.151
Transforming growth factor-beta-induced protein ig-h3	-0.026	0.070	-0.562	-0.261	-0.221	-0.188	0.100	0.208	0.809**	0.223
Vasorin	-0.252	-0.059	0.283	-0.357	-0.557	−0.815**	-0.142	-0.094	-0.512	0.602
Vitamin K-dependent protein S	-0.095	0.253	−0.696*	0.514	0.087	0.179	-0.119	0.249	0.575	0.242
Vitronectin	-0.162	−0.366*	0.150	-0.617	-0.340	−0.768**	-0.154	-0.091	-0.221	-0.365

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Appendix 5G – Correlations between HDL-associated proteins on S-HDL-P and HDL-CEC parameters in women and men across the groups

Table 5.16 *Proteins on small HDL that significantly correlated with total efflux in women and men.*

S-HDL-associated protein	All		Non-OB		T1DM		OB		T2DM	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
Albumin	-0.211	-0.090	-0.540	0.037	-0.027	0.046	-0.379	-0.151	−0.808**	0.096
Alpha-1-antichymotrypsin	0.104	0.345*	-0.420	0.311	0.179	0.521	0.083	0.303	-0.288	0.309
Alpha-1B-glycoprotein	0.033	0.342*	0.024	0.505	0.278	0.237	-0.249	0.325	-0.075	0.126
Alpha-2-HS-glycoprotein	-0.254	0.302	−0.690*	0.590	0.012	-0.207	-0.187	0.582	-0.229	0.113
Alpha-2-macroglobulin	0.029	-0.079	-0.162	0.185	-0.132	-0.214	0.195	-0.551	−0.726*	0.200
Apolipoprotein A-II	0.174	−0.332*	0.332	-0.522	0.222	-0.362	0.405	-0.064	0.620	-0.279
Apolipoprotein A-IV	-0.111	0.298	-0.126	-0.171	-0.090	0.492	0.155	0.008	−0.705*	0.430
Apolipoprotein C-I	0.001	0.336*	0.427	-0.230	-0.116	0.065	-0.195	0.584	0.586	0.487
Apolipoprotein C-II	0.045	0.128	0.646*	0.126	0.300	-0.198	-0.043	-0.136	0.583	0.246
Apolipoprotein C-III	0.155	0.293	0.403	0.136	0.339	0.320	0.051	0.752*	0.691*	0.408
Apolipoprotein C-IV	-0.172	0.014	0.286	-0.625	0.558	0.779*	-0.543	0.554	0.105	0.255
Apolipoprotein D	0.197	-0.280	0.650*	-0.272	0.128	-0.013	0.126	-0.349	0.644*	0.227
Beta-2-glycoprotein 1	0.166	0.169	0.112	0.040	0.178	0.523	0.726*	-0.352	0.036	0.113
Beta-Ala-His dipeptidase	0.219	0.220	0.492	-0.048	0.653*	0.033	-0.234	0.516	0.543	0.327
C3/C5 convertase	-0.153	0.481**	-0.522	0.377	0.233	0.670*	0.059	0.110	0.510	0.403
C4b-binding protein alpha chain	0.149	0.515***	-0.310	0.462	0.415	0.732*	-0.014	0.523	0.472	0.281
CD5 antigen-like	0.056	0.001	-0.196	0.149	-0.483	-0.277	0.677*	-0.451	0.119	0.811
Clusterin	0.219	0.149	0.652	0.023	0.105	-0.477	0.145	0.363	0.732*	0.212
Coagulation factor IX	−0.331*	0.351*	0.157	-0.311	0.175	0.250	−0.682*	0.632	-0.481	0.052
Coagulation factor V	-0.067	0.334*	0.169	0.134	0.019	-0.456	0.029	0.539	-0.304	0.826**
Coagulation factor X	0.196	0.381*	0.501	0.595	0.358	-0.092	-0.069	0.500	0.577	0.366
Coagulation factor XII	-0.008	0.211	0.018	0.273	0.080	0.696*	0.068	0.173	-0.090	0.187
Coagulation factor XIII B chain	0.349*	-0.170	0.575	0.029	0.104	-0.194	0.277	-0.513	0.559	0.340
Complement C1r subcomponent-like protein	−0.329*	0.026	0.115	-0.480	-0.347	-0.419	-0.588	0.065	-0.038	0.336
Complement C2	-0.230	0.373*	-0.572	-0.090	-0.376	0.707*	-0.295	0.376	0.304	-0.343
Complement C3	-0.098	-0.045	0.370	-0.375	0.264	-0.300	-0.581	0.260	0.845**	-0.128
Complement component C7	0.153	0.059	0.290	0.008	-0.493	−0.821**	0.333	0.147	0.577	0.127
Complement component C8 alpha chain	-0.184	0.441**	-0.473	0.537	0.265	0.938****	-0.056	-0.497	-0.563	0.701*
Complement component C8 beta chain	-0.149	0.391*	-0.500	0.621	0.456	0.856**	0.103	-0.496	−0.685*	0.564
Complement component C8 gamma chain	−0.430**	0.424**	−0.712*	0.636*	0.462	0.816**	-0.515	-0.357	-0.561	0.439
Complement component C9	-0.018	0.453**	0.008	0.292	0.387	0.713*	0.079	0.005	-0.599	0.646*
Complement factor H-related protein 1	0.416**	0.168	0.717*	0.001	0.491	0.194	0.161	-0.214	0.768**	0.330
Complement subcomponent C1r	-0.183	0.157	0.647*	-0.554	-0.482	-0.073	-0.451	0.333	0.144	0.166
Cysteine-rich secretory protein 3	0.079	−0.327*	0.393	-0.034	-0.548	-0.118	-0.161	−0.850**	-0.140	-0.106
EGF-containing fibulin-like extracellular matrix protein 1	0.156	0.195	-0.091	-0.258	0.181	0.713*	0.106	0.006	0.257	0.325
Endoplasmic reticulum chaperone BiP	-0.011	0.328*	0.333	0.146	-0.067	0.014	0.078	0.358	-0.119	0.447
Fibronectin	0.092	0.134	-0.139	0.692	0.272	-0.164	0.205	-0.184	−0.717*	0.270
Hemoglobin subunit beta	-0.338	0.027	0.173	0.334	0.457	-0.434	-0.567	−0.687*	-0.360	-0.062
Hemopexin	−0.315*	0.055	−0.822**	-0.043	-0.191	0.259	-0.135	0.081	-0.532	-0.215
Heparin cofactor 2	-0.128	0.445**	-0.239	0.603	0.561	0.163	-0.438	0.545	0.026	0.176
Hyaluronan-binding protein 2	0.005	0.303	-0.238	-0.190	0.164	0.459	0.128	-0.338	-0.105	0.715*
Immunoglobulin heavy constant gamma 3	0.130	−0.477**	0.113	-0.447	-0.577	−0.633*	0.379	-0.297	0.415	−0.693*
Immunoglobulin heavy variable 1-24	-0.050	-0.092	-0.130	0.428	-0.248	-0.224	-0.153	−0.694*	-0.028	-0.196
Immunoglobulin heavy variable 1-69	0.327*	0.005	0.550	0.089	-0.300	0.207	0.590	-0.278	0.134	0.211
Immunoglobulin heavy variable 2-70D	0.052	0.051	-0.151	0.127	0.729*	0.098	−0.689*	0.325	0.284	0.009
Immunoglobulin heavy variable 3-13	0.246	-0.292	-0.012	0.198	0.105	-0.310	0.642*	-0.166	0.197	-0.522
Immunoglobulin heavy variable 3-49	0.216	-0.017	-0.318	0.594	-0.460	0.384	0.775**	-0.187	0.365	-0.409
Immunoglobulin heavy variable 3-7	0.311	0.008	0.301	-0.050	-0.107	0.228	0.881***	0.105	0.002	-0.014
Immunoglobulin heavy variable 3-72	0.444**	-0.113	0.310	0.401	0.162	0.383	0.651*	-0.231	0.151	-0.107
Immunoglobulin heavy variable 3-73	0.322*	-0.101	0.338	0.450	-0.156	0.038	0.601	-0.066	0.714*	-0.075
Immunoglobulin heavy variable 3-74	0.250	-0.056	0.737*	0.340	-0.209	0.170	0.620	-0.044	-0.140	-0.225
Immunoglobulin heavy variable 4-38-2	0.427**	0.004	0.662*	-0.042	-0.101	0.437	0.509	-0.102	0.307	0.017
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	0.007	-0.194	0.123	-0.090	-0.292	0.180	0.025	−0.659*	-0.291	0.008
Immunoglobulin heavy variable 5-51	0.323*	-0.013	0.309	0.240	-0.075	0.610	0.577	-0.262	0.460	0.302
Immunoglobulin heavy variable 6-1	0.413**	0.169	0.711*	0.208	-0.353	-0.045	0.621	0.134	0.255	0.084
Immunoglobulin J chain	0.171	0.029	-0.333	0.170	0.435	0.149	0.368	-0.426	0.385	0.802*
Immunoglobulin kappa variable 1-17	0.162	0.143	0.160	0.143	-0.583	0.505	0.788**	0.430	0.273	0.182
Immunoglobulin kappa variable 1-33	0.198	-0.061	0.167	0.530	-0.400	0.211	0.229	-0.282	0.771**	-0.383
Immunoglobulin kappa variable 1-5	0.233	-0.079	-0.189	-0.095	-0.386	0.135	0.709*	-0.151	0.085	-0.098
Immunoglobulin kappa variable 2D-28	-0.127	-0.118	−0.636*	0.081	-0.332	0.239	0.005	0.123	0.042	-0.214
Immunoglobulin kappa variable 2D-29	0.352*	-0.241	-0.118	0.426	0.444	-0.006	0.545	-0.428	0.490	-0.578
Immunoglobulin kappa variable 4-1	0.289	-0.180	-0.077	0.042	-0.088	0.165	0.776**	-0.109	0.368	-0.492
Immunoglobulin lambda constant 3	0.170	−0.411**	0.614	-0.411	-0.326	-0.495	0.037	-0.392	0.380	0.131
Immunoglobulin lambda constant 7 (Fragment)	0.060	-0.160	-0.394	-0.339	-0.560	-0.238	0.717*	0.176	-0.164	-0.630
Immunoglobulin lambda variable 3-9	-0.018	-0.236	-0.027	0.309	-0.287	0.038	-0.094	−0.701*	0.237	-0.120
Immunoglobulin lambda variable 4-69	0.150	-0.235	-0.086	0.290	0.398	-0.276	0.489	0.054	-0.260	−0.736*
Immunoglobulin lambda variable 5-45 (Fragment)	-0.053	−0.330*	0.037	-0.274	-0.585	-0.554	-0.130	-0.244	0.459	−0.637*
Immunoglobulin lambda variable 8-61	0.391*	0.328*	0.182	0.370	0.236	0.637*	0.808**	0.266	0.287	-0.118
Immunoglobulin lambda-like polypeptide 5	0.048	−0.330*	0.059	0.292	-0.279	-0.550	0.004	-0.507	0.332	-0.105
Insulin-like growth factor-binding protein 3	-0.135	0.104	0.162	0.388	-0.001	−0.865**	-0.307	0.416	0.384	0.217
Insulin-like growth factor-binding protein complex acid labile subunit	-0.286	0.142	0.077	0.341	-0.567	0.076	-0.363	0.719*	-0.082	0.187
Keratin, type I cytoskeletal 9	0.463	0.353	0.848**	0.084	0.994	–	-0.942	0.128	0.387	0.486
Keratin, type II cytoskeletal 1	0.277	0.185	0.690*	0.154	0.180	0.318	0.034	0.095	-0.253	0.456
Kininogen-1	0.227	0.412**	0.567	0.536	-0.281	0.321	0.517	0.451	0.550	0.324
L-lactate dehydrogenase B chain	0.313	-0.108	0.636*	-0.448	0.333	-0.343	0.258	-0.629	-0.265	0.421
L-selectin	0.155	−0.359*	0.490	-0.207	-0.466	-0.592	0.101	-0.467	-0.008	0.087
Low affinity immunoglobulin gamma Fc region receptor III-A	0.083	-0.224	0.799**	0.078	0.200	-0.426	−0.634*	-0.424	0.060	0.130
Phosphatidylcholine-sterol acyltransferase	0.127	−0.357*	0.502	−0.707*	−0.717*	-0.310	0.057	−0.696*	0.715*	0.353
Plasma protease C1 inhibitor	-0.079	-0.045	0.178	-0.273	0.197	-0.020	0.076	0.027	−0.666*	-0.067
Probable non-functional immunoglobulin heavy variable 3-35	0.341	-0.392	0.475	-0.805	0.519	-0.473	-0.058	-0.224	0.307	−0.834*
Probable non-functional immunoglobulin heavy variable 3-38	0.334*	0.130	0.172	0.641*	0.335	0.670*	0.179	-0.233	0.368	0.068
Protein Z-dependent protease inhibitor	-0.012	0.260	0.382	-0.059	0.100	0.769**	-0.529	0.543	0.377	-0.509
SAA2-SAA4	-0.149	-0.022	-0.216	-0.272	0.311	-0.276	-0.392	0.694*	0.619	-0.242
Serum amyloid P-component	-0.031	0.222	0.439	0.528	0.125	0.290	-0.220	-0.577	-0.246	0.718*
Serum paraoxonase/arylesterase 1	0.042	0.039	0.621	-0.187	-0.125	-0.468	-0.167	0.694*	0.276	-0.155
SPARC	0.129	0.115	-0.051	0.204	0.296	0.664*	0.038	0.331	0.460	-0.627
Sulfhydryl oxidase 1	-0.013	0.000	0.300	-0.218	-0.501	-0.086	-0.119	0.312	0.635*	-0.064
Tetranectin	0.040	0.087	-0.105	0.631	0.068	-0.043	0.076	-0.301	-0.101	0.838**
Transthyretin	0.086	0.005	-0.502	0.727	-0.676	0.361	0.347	−0.734*	-0.281	-0.368
Vitamin K-dependent protein C	0.133	0.372*	-0.190	0.178	-0.233	0.508	0.219	0.487	0.675*	-0.085
Vitamin K-dependent protein S	0.189	0.142	0.005	-0.108	0.410	-0.213	0.056	0.477	0.708*	0.177

Correlation coefficient (r)

+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Table 5.17 *Proteins on small HDL that significantly correlated with ABCA1-dependent CEC in women and men.*

S-HDL-associated protein	All		Non-OB		T1DM		OB		T2DM	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
Alpha-1-antitrypsin	0.138	0.201	-0.272	0.714*	0.115	-0.075	0.418	-0.126	-0.640	-0.075
Alpha-1B-glycoprotein	-0.166	0.363*	-0.021	0.641*	-0.249	0.297	-0.544	0.380	-0.014	0.195
Alpha-2-antiplasmin	−0.385*	0.115	-0.430	-0.134	-0.548	-0.060	-0.367	0.565	-0.297	-0.391
Antithrombin-III	0.182	0.218	-0.229	0.732*	0.351	-0.049	0.707*	-0.378	-0.651	0.116
Apolipoprotein A-II	0.034	-0.277	0.338	−0.673*	-0.559	-0.200	0.062	0.049	0.807**	-0.333
Apolipoprotein B-100	0.164	0.153	0.593	0.682*	-0.177	0.655*	-0.231	-0.434	0.066	-0.031
Apolipoprotein C-I	-0.148	0.422**	0.190	-0.374	-0.628	0.124	-0.304	0.738*	0.338	0.549
Apolipoprotein C-III	0.004	0.361*	0.258	-0.096	-0.341	0.401	-0.160	0.782**	0.172	0.617
Apolipoprotein C-IV	-0.094	-0.066	0.416	-0.756	-0.035	0.789*	-0.631	0.303	-0.498	0.035
Apolipoprotein D	0.100	−0.405**	0.618	-0.508	-0.525	0.142	0.000	-0.430	0.712*	-0.246
Apolipoprotein E	-0.202	0.325*	-0.199	-0.497	-0.106	-0.328	-0.190	0.267	-0.236	0.722*
Apolipoprotein L1	-0.169	-0.134	-0.047	−0.635*	0.042	-0.140	-0.267	-0.406	0.340	-0.200
Beta-2-glycoprotein 1	0.290	0.081	0.355	-0.037	0.449	0.325	0.839**	-0.131	0.065	-0.277
Beta-Ala-His dipeptidase	0.062	0.443**	0.137	-0.149	0.092	0.274	-0.478	0.656*	0.139	0.732*
C3/C5 convertase	-0.178	0.422	-0.113	0.410	-0.507	0.446	0.066	-0.027	0.367	0.422
C4b-binding protein alpha chain	-0.124	0.474**	-0.189	0.340	-0.340	0.610	-0.179	0.383	-0.161	0.469
CD5 antigen-like	0.039	-0.052	-0.376	0.208	-0.361	0.029	0.805**	-0.545	0.747	0.161
Coagulation factor IX	-0.281	0.347*	0.157	-0.421	-0.274	0.171	-0.541	0.639*	-0.104	0.240
Coagulation factor X	0.018	0.400*	0.306	0.498	-0.440	0.005	-0.124	0.737*	0.234	-0.060
Coagulation factor XII	0.112	0.135	0.334	0.309	0.642*	0.643*	-0.188	0.128	-0.515	-0.298
Complement C1r subcomponent-like protein	−0.339*	-0.122	0.137	-0.394	-0.548	-0.335	−0.730*	-0.049	0.313	-0.274
Complement C1s subcomponent	-0.119	−0.385*	-0.109	−0.784**	-0.374	-0.562	-0.028	-0.066	0.152	-0.138
Complement C2	−0.315*	0.297	-0.417	0.014	−0.698*	0.552	−0.706*	0.311	0.432	-0.171
Complement C5	0.021	0.085	0.199	−0.656*	-0.016	-0.264	-0.259	0.043	0.387	0.378
Complement component C7	0.116	0.044	-0.032	-0.210	-0.494	−0.710*	0.362	0.158	0.647	0.258
Complement component C8 alpha chain	-0.204	0.236	-0.062	0.313	0.178	0.841**	-0.038	−0.649*	-0.645	0.256
Complement component C8 beta chain	-0.140	0.173	-0.091	0.479	0.023	0.734*	0.248	−0.647*	−0.753*	0.064
Complement component C8 gamma chain	−0.414**	0.263	-0.238	0.666*	0.354	0.625	-0.390	-0.393	-0.508	0.094
Complement factor H	0.137	-0.110	0.074	−0.665*	0.166	-0.276	-0.509	-0.501	0.825**	0.090
Complement factor H-related protein 1	0.378*	0.060	0.394	0.096	0.115	-0.023	0.028	-0.364	0.832**	0.148
Complement factor H-related protein 2	0.332*	-0.022	0.376	0.011	0.124	0.479	0.231	-0.567	0.186	-0.216
Complement subcomponent C1r	-0.066	0.037	0.756*	-0.418	-0.238	-0.171	-0.605	0.260	-0.006	-0.221
Cysteine-rich secretory protein 3	0.055	-0.285	0.177	-0.105	-0.477	-0.003	-0.219	−0.650*	0.145	-0.057
EGF-containing fibulin-like extracellular matrix protein 1	0.029	0.082	-0.362	-0.373	-0.320	0.736*	0.025	0.007	0.063	-0.002
Fibulin-1	0.368*	-0.279	0.700*	-0.145	0.724	-0.115	0.106	-0.351	-0.157	-0.477
Hemoglobin subunit alpha	-0.169	0.098	-0.425	0.269	0.358	−0.689*	-0.509	-0.582	-0.668	0.333
Hemoglobin subunit beta	-0.253	0.148	-0.376	0.241	0.524	−0.643*	-0.522	-0.541	-0.763	0.306
Heparin cofactor 2	-0.108	0.405**	0.030	0.830**	0.402	0.005	-0.500	0.476	-0.433	0.030
Hepatocyte growth factor activator	0.157	-0.116	0.503	0.674*	0.258	-0.414	-0.047	-0.226	-0.652	-0.289
Histidine-rich glycoprotein	-0.195	0.083	−0.849**	-0.076	-0.158	−0.647*	-0.059	0.587	0.489	-0.125
Immunoglobulin heavy constant gamma 3	0.149	−0.477**	-0.152	-0.541	0.014	-0.358	0.105	-0.398	0.479	-0.585
Immunoglobulin heavy constant mu	0.145	0.279	0.011	0.360	-0.231	0.004	0.415	-0.314	-0.435	0.693*
Immunoglobulin heavy variable 1-24	0.032	-0.070	-0.448	0.428	0.275	0.114	-0.040	−0.692*	-0.025	-0.079
Immunoglobulin heavy variable 1-46	0.331*	0.043	0.245	-0.332	0.396	0.489	0.390	-0.594	0.026	0.124
Immunoglobulin heavy variable 1-69	0.483**	-0.063	0.499	-0.020	0.234	0.387	0.631	-0.438	-0.111	-0.033
Immunoglobulin heavy variable 2-26	0.192	-0.014	0.078	-0.096	0.690*	0.548	-0.753	-0.093	0.205	-0.168
Immunoglobulin heavy variable 2-70D	0.069	0.083	-0.446	0.118	0.773*	0.504	-0.319	0.221	0.458	-0.070
Immunoglobulin heavy variable 3-15	0.076	0.113	-0.392	-0.104	0.024	0.371	0.280	-0.320	-0.568	0.640*
Immunoglobulin heavy variable 3-49	0.167	-0.149	-0.471	0.537	-0.094	0.434	0.740*	-0.407	-0.112	−0.709*
Immunoglobulin heavy variable 3-7	0.324*	-0.025	-0.064	-0.006	0.187	0.260	0.948***	-0.097	-0.389	0.117
Immunoglobulin heavy variable 3-74	0.363*	-0.064	0.694*	0.327	0.272	0.242	0.659	-0.225	-0.425	-0.082
Immunoglobulin heavy variable 4-38-2	0.414**	-0.058	0.352	0.055	0.441	0.329	0.442	-0.338	-0.008	0.158
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	0.024	-0.149	-0.104	-0.264	0.366	0.350	-0.130	−0.635*	−0.762*	0.260
Immunoglobulin heavy variable 5-51	0.354*	-0.054	0.126	0.092	0.468	0.716*	0.244	-0.512	0.396	0.542
Immunoglobulin kappa variable 1-17	0.138	0.181	-0.235	0.029	-0.207	0.668*	0.713*	0.207	0.283	0.643*
Immunoglobulin kappa variable 1-5	0.256	-0.140	-0.524	-0.157	-0.094	0.223	0.745*	-0.476	0.209	0.238
Immunoglobulin kappa variable 1-8	-0.026	-0.082	-0.451	0.682*	-0.087	0.109	0.244	-0.606	−0.795*	-0.191
Immunoglobulin kappa variable 2D-28	-0.128	-0.015	−0.693*	0.024	0.077	0.342	-0.128	0.274	-0.048	0.077
Immunoglobulin kappa variable 3D-15	0.173	-0.211	-0.481	-0.153	0.224	0.379	0.408	−0.665*	0.666	0.308
Immunoglobulin lambda variable 2-14	-0.146	0.008	-0.534	0.100	−0.724*	0.381	0.196	-0.343	-0.703	0.304
Immunoglobulin lambda variable 3-16	-0.011	0.007	-0.324	0.542	-0.489	0.270	0.230	-0.459	0.898**	-0.358
Immunoglobulin lambda variable 3-9	-0.072	-0.288	-0.479	0.076	0.070	0.289	-0.086	−0.764*	0.058	-0.096
Immunoglobulin lambda variable 4-69	0.110	-0.173	-0.163	0.377	0.099	-0.124	0.603	0.136	0.410	−0.669*
Immunoglobulin lambda variable 8-61	0.274	0.139	0.122	0.066	-0.328	0.313	0.706*	0.144	0.244	-0.120
Immunoglobulin lambda variable 9-49	0.283	-0.147	-0.817	-0.383	0.398	-0.311	0.802*	-0.011	-0.121	-0.585
Insulin-like growth factor-binding protein complex acid labile subunit	−0.354*	0.192	0.010	0.316	−0.841**	0.153	-0.534	0.872**	0.377	0.030
Inter-alpha-trypsin inhibitor heavy chain H1	0.151	-0.036	-0.128	0.677*	0.330	-0.271	0.114	-0.597	-0.395	-0.208
Inter-alpha-trypsin inhibitor heavy chain H2	0.240	0.014	-0.084	0.697*	0.457	-0.295	0.298	-0.160	-0.389	-0.411
Kininogen-1	0.179	0.411**	0.629	0.717*	-0.212	0.283	0.350	0.479	0.432	-0.124
L-selectin	0.270	−0.400*	0.407	-0.107	-0.237	-0.491	0.220	-0.605	-0.266	-0.133
Low affinity immunoglobulin gamma Fc region receptor III-A	0.186	-0.278	0.814**	0.048	-0.052	-0.593	-0.507	-0.497	0.229	-0.276
Lumican	0.058	0.209	0.203	0.543	−0.744*	0.115	-0.173	-0.042	0.186	0.320
N-acetylmuramoyl-L-alanine amidase	-0.159	0.250	-0.259	0.376	0.072	0.320	-0.294	0.713*	-0.210	-0.369
Phosphatidylinositol-glycan-specific phospholipase D	0.263	0.103	0.206	0.684*	0.219	-0.274	0.401	-0.033	0.637	0.060
Platelet factor 4	-0.117	0.287	-0.189	0.310	−0.765*	0.221	0.116	0.040	0.421	0.363
Protein Z-dependent protease inhibitor	-0.088	0.132	0.332	-0.384	−0.635*	0.583	−0.698*	0.621	-0.133	−0.697*
SAA2-SAA4	-0.297	0.047	-0.025	-0.067	-0.579	-0.102	-0.661	0.692*	0.780*	-0.223
Serum amyloid P-component	0.038	0.350*	0.430	0.681*	0.172	0.384	-0.045	-0.454	-0.463	0.698*
Serum paraoxonase/arylesterase 1	-0.083	0.042	0.306	-0.243	-0.419	-0.337	-0.411	0.677*	0.138	-0.229
SPARC	-0.069	0.177	-0.117	0.536	-0.396	0.717*	0.002	0.314	0.192	-0.360
Vitamin K-dependent protein C	0.265	0.372*	0.002	0.455	0.102	0.297	-0.157	0.401	0.423	-0.014
Vitronectin	-0.188	0.180	0.141	-0.570	0.094	-0.142	-0.594	0.692*	-0.545	0.006

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 5.18 *Proteins on small HDL that significantly correlated with ABCA1-independent efflux in women and men.*

S-HDL-associated protein	All		Non-OB		T1DM		OB		T2DM	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
Actin, cytoplasmic 1	0.254	-0.131	0.501	-0.248	0.718*	0.589	-0.351	0.198	0.471	-0.491
Afamin	0.085	-0.222	-0.068	-0.169	0.762*	-0.448	-0.182	0.171	0.686*	-0.075
Alpha-1-antichymotrypsin	-0.089	0.427**	-0.536	0.126	-0.174	0.502	-0.024	0.280	0.518	0.618
Angiotensinogen	0.145	0.303	−0.716*	0.057	0.271	0.282	0.632	0.242	0.244	0.349
Apolipoprotein A-I	0.119	-0.144	0.121	-0.480	0.399	−0.702*	0.577	0.294	-0.356	0.246
Apolipoprotein A-II	0.232	-0.265	-0.019	-0.151	0.796**	-0.430	0.652*	-0.205	-0.161	0.044
Apolipoprotein A-IV	-0.205	0.345*	-0.278	0.133	-0.422	0.569	0.211	0.298	-0.047	0.213
Apolipoprotein C-III	0.244	0.089	0.190	0.388	0.686*	0.117	0.039	0.484	0.458	-0.220
Apolipoprotein D	0.173	-0.012	0.024	0.122	0.668*	-0.190	-0.149	-0.131	0.009	0.585
Apolipoprotein E	-0.028	-0.055	0.643*	0.225	0.017	-0.133	-0.569	-0.282	0.157	-0.158
C3/C5 convertase	0.008	0.359*	-0.555	0.206	0.753*	0.705*	0.047	0.270	0.351	0.009
C4b-binding protein alpha chain	0.418**	0.357*	-0.159	0.461	0.799**	0.626	-0.193	0.562	0.804**	-0.205
Ceruloplasmin	0.285	0.175	-0.143	-0.047	0.163	0.588	0.707*	-0.017	-0.409	-0.103
Coagulation factor IX	-0.132	0.214	-0.005	-0.064	0.455	0.258	−0.723*	0.432	0.083	-0.221
Coagulation factor V	0.070	0.363*	0.145	0.338	0.675	-0.538	-0.354	0.595	-0.070	0.566
Coagulation factor X	0.290	0.203	0.256	0.518	0.807**	-0.176	-0.556	0.017	0.628	0.539
Complement C1r subcomponent-like protein	-0.051	0.199	-0.035	-0.426	0.218	-0.375	-0.372	0.206	-0.020	0.757*
Complement C2	0.079	0.317*	-0.198	-0.191	0.350	0.648*	-0.144	0.355	-0.159	-0.233
Complement component C7	0.080	0.053	0.440	0.285	0.025	−0.674*	-0.301	0.088	-0.241	-0.149
Complement component C8 alpha chain	-0.005	0.515***	-0.559	0.642*	0.078	0.734*	0.268	-0.135	0.267	0.588
Complement component C8 beta chain	-0.040	0.502***	-0.556	0.592	0.426	0.710*	-0.040	-0.137	0.142	0.643*
Complement component C8 gamma chain	-0.101	0.451**	−0.638*	0.381	0.090	0.763*	-0.039	-0.200	0.266	0.447
Complement component C9	-0.001	0.522***	-0.110	0.160	0.138	0.677*	0.094	0.238	-0.019	0.571
Complement factor I	-0.262	0.346*	−0.744*	0.279	-0.225	0.642*	0.307	0.105	0.184	0.445
Cysteine-rich secretory protein 3	0.051	-0.246	0.289	0.068	-0.070	-0.213	-0.252	−0.877***	-0.290	-0.067
Endoplasmic reticulum chaperone BiP	0.023	0.369*	0.643	0.360	-0.062	0.230	-0.263	0.561	-0.114	0.467
Fibrinogen alpha chain	0.143	0.058	0.081	0.166	0.772*	0.698*	-0.137	0.146	0.154	-0.303
Fibronectin	0.300	0.132	-0.122	0.169	0.414	0.199	0.742*	-0.081	0.030	0.328
Gelsolin	-0.231	0.231	−0.716*	0.190	-0.310	0.313	0.270	0.074	0.502	0.617
Hemoglobin subunit alpha	0.012	-0.129	0.721*	0.517	-0.043	-0.154	-0.683	-0.524	-0.036	-0.435
Hemoglobin subunit beta	-0.194	-0.133	0.741*	0.341	-0.098	-0.043	-0.619	−0.691*	0.028	-0.446
Hemopexin	-0.111	0.126	−0.674*	-0.241	-0.188	0.026	0.377	0.107	0.048	0.409
Heparin cofactor 2	-0.052	0.313*	-0.368	0.108	0.137	0.292	-0.282	0.478	0.543	0.189
Hepatocyte growth factor activator	-0.137	0.227	-0.124	0.123	-0.182	0.281	-0.419	-0.172	0.252	0.823*
Hyaluronan-binding protein 2	-0.051	0.377*	-0.412	0.085	0.083	0.458	0.285	-0.333	-0.152	0.824*
Immunoglobulin heavy constant gamma 1	-0.066	-0.281	0.450	-0.024	−0.710*	−0.636*	-0.059	-0.168	-0.514	-0.140
Immunoglobulin heavy constant gamma 3	-0.003	-0.284	0.366	-0.175	-0.583	−0.738*	0.244	-0.067	-0.253	-0.182
Immunoglobulin heavy variable 1-18	-0.055	-0.247	0.507	0.096	−0.766**	-0.213	-0.172	-0.202	-0.129	−0.634*
Immunoglobulin heavy variable 2-26	-0.058	0.258	-0.006	-0.254	-0.641	-0.082	0.644	0.172	-0.382	0.801*
Immunoglobulin heavy variable 3-13	0.292	−0.360*	0.152	0.349	0.469	−0.743*	0.658*	-0.306	-0.502	−0.711*
Immunoglobulin heavy variable 3-15	0.163	-0.041	0.276	0.142	-0.246	0.060	0.295	0.103	0.646*	-0.432
Immunoglobulin heavy variable 3-72	0.503***	-0.161	0.761*	0.371	-0.064	-0.007	0.803**	0.150	0.004	-0.624
Immunoglobulin heavy variable 3/OR16-12 (non-functional) (Fragment)	0.376*	-0.078	0.716*	-0.374	0.592	0.020	-0.209	0.055	0.129	-0.071
Immunoglobulin heavy variable 4-4	-0.074	0.094	0.541	0.265	−0.738*	-0.109	0.286	0.500	0.325	-0.194
Immunoglobulin heavy variable 4/OR15-8 (non-functional) (Fragment)	-0.023	-0.171	0.313	0.162	−0.697*	-0.080	0.067	-0.494	0.230	-0.302
Immunoglobulin heavy variable 6-1	0.298	0.053	0.399	-0.012	-0.058	0.036	0.786**	0.332	-0.045	-0.410
Immunoglobulin kappa constant	0.139	-0.243	0.802**	0.181	-0.080	-0.394	-0.019	-0.114	-0.218	-0.534
Immunoglobulin kappa variable 1-33	0.004	0.130	0.432	0.677*	-0.492	0.130	-0.045	0.036	0.255	-0.010
Immunoglobulin kappa variable 1-8	0.275	-0.170	0.827**	0.196	0.175	0.083	-0.023	-0.372	0.228	-0.371
Immunoglobulin kappa variable 1D-39	-0.054	-0.100	0.712*	-0.139	-0.693	-0.752	-0.610	0.158	0.451	-0.381
Immunoglobulin kappa variable 2-30	0.324*	-0.019	0.697*	0.230	0.154	0.134	0.123	0.240	0.583	-0.511
Immunoglobulin kappa variable 2D-29	0.436**	-0.209	0.689*	0.213	0.250	0.195	0.542	-0.388	0.105	-0.398
Immunoglobulin kappa variable 3-11	0.062	0.058	0.318	-0.061	-0.303	0.259	-0.133	0.298	0.884***	-0.087
Immunoglobulin kappa variable 3-20	0.371*	-0.135	0.848**	0.138	-0.196	-0.061	0.493	0.138	0.236	-0.630
Immunoglobulin kappa variable 6-21	0.331*	0.025	-0.317	0.453	0.638	0.202	0.112	-0.315	-0.247	-0.152
Immunoglobulin lambda constant 3	0.041	−0.446**	0.722*	-0.074	-0.372	−0.697*	-0.088	-0.412	-0.201	-0.394
Immunoglobulin lambda variable 1-51	0.232	0.070	0.757*	0.559	-0.256	-0.312	-0.053	0.202	-0.063	0.085
Immunoglobulin lambda variable 2-11	0.112	-0.114	0.742*	-0.660	-0.346	0.253	0.173	-0.092	-0.221	-0.192
Immunoglobulin lambda variable 3-19	-0.058	0.177	-0.201	0.134	-0.188	0.194	-0.255	-0.042	0.695*	0.348
Immunoglobulin lambda variable 3-9	0.075	-0.077	0.633*	0.503	-0.377	-0.265	-0.165	-0.401	0.405	-0.039
Immunoglobulin lambda variable 5-45 (Fragment)	0.085	−0.383*	0.515	-0.183	-0.184	-0.373	0.206	-0.510	0.029	-0.559
Immunoglobulin lambda variable 7-46	0.377*	-0.166	0.258	0.392	0.591	-0.851	0.301	-0.381	-0.017	0.302
Immunoglobulin lambda variable 8-61	0.243	0.427**	0.078	0.633*	0.597	0.798**	0.455	0.355	-0.311	-0.006
Immunoglobulin lambda variable 9-49	-0.115	0.153	0.647	-0.237	-0.505	0.022	0.023	0.169	0.862**	0.641
Insulin-like growth factor-binding protein 3	0.043	-0.010	0.604	0.577	0.277	−0.861**	-0.123	0.165	0.157	-0.059
Inter-alpha-trypsin inhibitor heavy chain H3	-0.238	0.360*	-0.242	0.239	-0.552	0.586	-0.073	-0.030	-0.402	0.250
ITIH4 protein	0.153	0.234	0.213	0.258	0.557	0.716*	-0.238	0.315	-0.287	-0.580
Kallistatin	-0.221	0.159	-0.083	−0.679*	−0.699*	-0.122	0.128	0.312	0.222	0.410
Keratin, type I cytoskeletal 10	0.506**	0.141	0.693*	0.271	0.426	-0.047	-0.388	-0.271	0.635*	0.710*
Keratin, type II cytoskeletal 1	0.274	0.320*	0.499	0.427	0.579	0.129	-0.050	0.169	0.607	0.701*
L-lactate dehydrogenase B chain	0.375*	-0.206	0.145	-0.148	0.596	-0.511	0.398	-0.462	0.354	-0.029
Phosphatidylcholine-sterol acyltransferase	-0.092	−0.325*	0.526	−0.697*	-0.528	-0.299	-0.449	−0.764*	0.377	0.377
Plasma kallikrein	0.265	0.017	-0.106	0.489	0.769**	0.013	0.319	-0.443	-0.173	-0.149
Plasminogen	-0.106	0.331*	-0.418	0.161	-0.499	0.563	0.454	0.312	0.437	0.240
Platelet factor 4	0.332*	0.191	0.513	0.319	0.758*	0.033	-0.179	0.727*	-0.205	-0.185
Protein Z-dependent protease inhibitor	0.106	0.313*	0.058	0.379	0.754*	0.727*	-0.428	0.271	0.549	0.186
SAA2-SAA4	0.184	-0.098	-0.260	-0.440	0.904***	-0.385	0.148	0.489	0.031	-0.041
SPARC	0.293	-0.008	0.094	-0.255	0.732*	0.379	0.069	0.257	0.240	-0.368
Tetranectin	-0.170	0.076	-0.168	0.673*	-0.125	0.002	-0.395	-0.216	0.091	0.389
Vitamin K-dependent protein Z	0.605	0.194	0.825*	0.483	0.368	-0.064	0.582	0.589	0.299	0.640

Correlation coefficient (r)
+ 1 Perfect
+ 0.800 - 0.999 Very strong
+ 0.600 - 0.799 Strong
+ 0.400 - 0.599 Moderate
+ 0.200 - 0.399 Weak
+ 0.000 - 0.199 Very Weak
Non-sig.
−0.000 - −0.199 Very Weak
−0.200 - −0.399 Weak
−0.400 - −0.599 Moderate
−0.600 - −0.799 Strong
−0.800 - −0.999 Very strong
−1 Perfect

Statistical significance is presented as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Chapter 6
General Discussion

6.1 General Discussion

The main objective of this thesis was to a gain greater understanding of HDL-P biology, and in particular the relationship between HDL-CEC and HDL protein composition, in people with and without T1DM, T2DM and obesity. Diabetes is one of the fastest growing global health emergencies of the 21st century. Today, more than half a billion people are living with diabetes worldwide [1]. Morbidity and mortality due to diabetes are mostly attributable to an elevated risk of CVD, which accounts for between one-third and one-half of all deaths [2]. HDL-P exert numerous atheroprotective functions [3], but its role in stimulating cholesterol efflux from peripheral cells, the first step of the reverse cholesterol transport pathway, is widely considered the dominant atheroprotective function of the particles. The proteins identified on HDL generally align with the assigned functions of the particles indicative that measuring HDL associated proteins may serve as novel biomarkers of HDL function. However, to date there has been a dearth of information on the ability of the HDL proteome to predict HDL-CEC. Hence, in Chapter 3 we set out to investigate the relationship between HDL-CEC and HDL-associated proteins, and furthermore developed novel biomarker panels and a scoring algorithm for total CEC, ABCA1-dependent CEC and ABCA1-independent CEC. Considering the alarmingly high levels of diabetes worldwide, we subsequently investigated the impact of T1DM, T2DM and obesity on HDL efflux capacity, particle size and HDL sub-fraction proteomic composition within Chapter 4, to gain greater understanding of the residual CVD risk present in these high-risk populations that is not attributable to traditional risk factors. Lastly, in Chapter 5, we examined sex-specific differences in HDL protein composition and function in people with and without T1DM, T2DM and obesity to gain greater insight into potential mechanisms driving the loss of female 'advantage' against CVD in the setting of diabetes. Our findings have collectively demonstrated that the HDL proteome tightly aligns with HDL-CEC, but proteins governing HDL-CEC differ in those with and without diabetes, and also differs between men and women. We have further demonstrated that measuring the proteomic composition of small versus large HDL-Ps, and in turn the function of small and large HDL-Ps, was critical to truly untangle the complex relationship between HDL structure and function.

HDL-C has been shown to be a robust, consistent, and independent predictor of CVD [4, 5]. However, pharmacologically raising HDL-C levels using CETP inhibitors failed to deliver an anti-atherogenic effect [6-9]. In parallel, GWAS studies failed to identify a link between genetically low HDL-C and increased risk of CVD [10-12]. This double hit to the HDL field highlighted that measurement of static HDL-C is likely an oversimplified estimate of HDL biology and questioned whether HDL is indeed causal in protecting against CVD or merely a biomarker of disease risk. The field soon turned to monitoring measures of HDL quality, over

measures of HDL-C quantity, including measurement of HDL-CEC, and identified that HDL-CEC was in fact a better predictor of CVD risk than HDL-C levels [13-15]. However, the relationship between HDL function and HDL composition is poorly understood and was therefore a major area of focus of this thesis. In particular, this thesis focused on the relationship between HDL-CEC and HDL-associated proteins.

The HDL proteome has long been debated as either a 'pot of gold' or Pandora's Box as discussed by Reilly and Tall in 2007 [16]. At that time it was hard to envisage that measuring all HDL-associated proteins (or key proteins) in a biomarker panel would be a possibility to comprehensively assess CVD risk. However, the biomarker field is rapidly evolving and with the emergence of targeted proteomics assays and accompanying data analytics capacity, it is entirely feasible that the HDL proteome will eventually emerge victorious in this debate. 251 proteins have been shown to be associated with HDL-P [17], and the HDL proteome is known to be effected in end stage renal disease [18], CVD [19, 20] and diabetes [20].

However, the causal relationship between the HDL proteome and associated functions of the particles is a major challenge in the HDL field. We have explored the relationship between the HDL proteome and HDL-CEC within this thesis in non-obesity, T1DM, T2DM and obesity groups, in individuals with high-HDL-CEC versus low-HDL-CEC and between men and women. However, it is important to note that we cannot infer a causal role for the proteins in driving HDL-CEC. This will be a major hurdle to overcome in future years – recombinant HDL enriched with proteins of interest may offer one mechanism to establish whether such proteins that are associated with HDL-CEC, can actually mediate CEC *in vitro*. Such mechanistic studies were beyond the scope of the current thesis but certainly are an area worthy of future investigation. Another major challenge in the HDL field is that there are numerous methods to isolate HDL, and within any given method there are also numerous sub-species of HDL with very different proteomes as was demonstrated within this thesis. This additional layer of complexity was important to uncover as the relationship between the HDL proteome and HDL-CEC was much stronger when the particles were split into large and small particles. This was an arbitrary decision on our behalf as to what constituted small (to the right of the FPLC HDL peak) and large (to the left of the FPLC peak) particles. In theory, we could have run HDL proteomics on 10-15 subfractions derived from FPLC (as performed by Gordon *et al*.) [21] and found even stronger relationships to functionality. Greater understanding of HDL sub-particle function and proteomic/lipidomic composition will be essential to gain further understanding as to whether HDL is indeed just one particle or a family of multiple ApoA-I containing particles with very differing functions and compositions that to date have been collated under an over-simplistic HDL particle umbrella.

Measuring HDL-CEC within a cell-culture model has emerged as a novel target and more vital determinant of CVD risk than static HDL-C levels [13-15], however, as detailed in the discussion sections of the thesis chapters the cell-based measures of HDL-CEC are complex and low-throughput. The assays are not standardized, and there is wide variation in the method conducted in the literature between different laboratory groups e.g. different cell-lines of macrophages used (murine J774s [13-15, 22] or human THP-1 cells [23, 24]), and different ways of assessing cholesterol efflux (fluorescence-labelled cholesterol [15], colorimetric assay [24], radiolabelled cholesterol [13, 14, 22, 23]), which make it difficult to cross compare studies. Hence, there is a major unmet need to identify robust biomarkers of HDL-CEC in order to develop new more efficient methods of predicting incidence of and/or progression of CVD. HDL proteomics is one approach to biomarker discovery, which could enable large-scale studies with a method that is more suitable for clinical use. Measuring HDL-proteins is semi-quantitative, quicker, higher throughput, reproducible and reliable and in general we observe less within-group variation within the HDL proteome relative to HDL-CEC. Currently, the McGillicuddy laboratory is developing a targeted proteomics array based on HDL-associated proteins with an accompanying novel high-throughput method to isolate HDL from serum which are key steps to realising the potential translation of HDL proteomics into clinical application. This thesis provides key data on the proteins that are associated with HDL-CEC in people with and without metabolic disease, that will be incorporated into the targeted proteomics assay. The data from this thesis has highlighted that specific sub-panels of biomarkers will be critical to develop for people with and without diabetes (with differing panels for T1DM versus T2DM). In addition, this thesis has highlighted that different biomarker panels for men and women will be required to ensure CVD risk is assessed in a comprehensive and stratified approach. However, future studies are needed to validate our findings in larger cohorts.

In Chapter 3, we first sought to investigate the relationship between HDL-associated proteins on large and small HDL-P combined (total HDL proteome) and HDL-CEC in people with high vs. low HDL-CEC. It became clear that different HDL proteins govern total CEC, ABCA1-dependent CEC and ABCA1-independent CEC, which formed the basis for the development of novel biomarker panels and risk-scores for total CEC, ABCA1-dependent CEC and ABCA1-independent CEC. It is notable in Chapter 3 that the correlations between individual HDL-proteins and HDL-CEC were in the range of ±0.2-0.4 (weak to moderate). However, upon combining the HDL-associated proteins that significantly correlated with HDL-CEC into a scoring algorithm, we demonstrate strong to very strong correlations with total CEC, ABCA1-dependent CEC, and ABCA1-independent CEC. Proteomics analysis revealed that even though there were only subtle differences observed between high vs. low effluxers (only two HDL-associated proteins met the threshold of the FDR) collectively they can be powerful

predictors; no one protein is a powerful a biomarker, hence it is essential to look at combinations of HDL-associated proteins. It's important to note that even if individual HDL-associated proteins are not significant/ don't meet the FDR threshold which adjusts for multiple comparisons, it does not necessarily mean that they are not important contributors to biomarker discovery. Indeed, our novel scores for HDL-CEC (ROC AUC of 0.859, *P*=0.0001*** for total efflux score, 0.862, *P*=0.0001*** for ABCA1-dependent efflux score and 0.781, *P*=0.0002** for ABCA1-independent efflux score), highlighted the promising potential of the HDL proteome to accurately differentiate between high vs. low HDL effluxers. Additionally, our score performs better than reported findings by Jin *et al.,* who developed a targeted proteomic panel and associated algorithm from the HDL-associated proteins, ApoA-I, ApoC-I, ApoC-II, ApoC-III and ApoC-IV and investigated the predictive capacity for total CEC (ROC AUC=0.62) [25]. However, while the HDL algorithmic scores we have generated may serve as surrogate biomarkers for the HDL-CEC parameters, future studies are needed to replicate and subsequently validate our findings in larger cohorts and to determine if the efflux scores can predict future CVD events and therefore those most at risk of CVD. Additionally, there is residual CEC that is likely dictated by other measures of HDL-P composition not captured in the proteomics efflux scores including lipidomic composition, particle size, post-translational lipid modifications and/or post-translational protein modifications which needs further investigation. Investigating all these aspects of HDL and combining them into a score/algorithm using machine learning/artificial intelligence will be an important future direction for this work. A major limitation of Chapter 3 was the collation of the HDL proteome into one fraction – it became apparent in the subsequent chapters that separation of the particles into large and small particles yielded much stronger correlations to HDL-CEC than the total proteome combined. Future directions investigating large, medium and small sized particles and relationship to CEC will be important to elucidate. In addition, it is notable in all the Chapters that there were weak associations between the proteome of S-HDL-P and ABCA1-dependent efflux, with stronger associations to ABCA1-independent efflux. These findings suggest that the particles we analysed are more likely acceptors via ABCA1-independent pathways, and given these particles are already lipidated (as evidenced by cholesterol accumulation in FPLC curves), we may not be capturing the particles driving ABCA1-dependent CEC. Further analysis of the smallest HDL fractions may be required to interrogate the proteins governing ABCA1-dependent CEC in more detail.

In Chapter 4, we profiled the HDL proteome of large and small HDL-P in people with and without T1DM or T2DM and conducted correlation analysis to the three different HDL-CEC parameters. We initially combined all groups together to compare the proteome of L-HDL-P and S-HDL-P and found profound differences in the HDL proteome across the different sized

particles (*n*=205 proteins reached significance after FDR). This further justified our approach for analysing the proteomic composition of the particles separately. It was notable that when all groups were combined into one group, there were mostly weak associations between proteins on both L-HDL-P and S-HDL-P and HDL-CEC parameters. However, correlations between the HDL-associated proteins on both L-HDL-P and S-HDL-P and HDL-CEC parameters increased to moderate and strong, when separated into the individual groups, indicative that different proteins govern HDL-CEC in people with and without metabolic disease. We consistently found strong associations between the concentrations of L-HDL-P and HDL-CEC (particularly total and ABCA1-independent CEC) and in turn the proteome of L-HDL-P strongly correlated with HDL-CEC, especially ABCA1-independent CEC. Our findings highlight that L-HDL-P, and its associated proteome, are likely important drivers of HDL-CEC in people with and without T1DM or T2DM. However, our findings also highlight that different proteins are associated with HDL-CEC conditional on metabolic disease status. For instance, in T1DM the correlations between ABCA1-independent CEC and ApoA-I, ApoA-II, Apo-IV, ApoC-I, ApoD and ApoE on L-HDL-P, evident in non-obese controls, were lost and ApoM (r=0.718) became the dominant apolipoprotein that positively associated with ABCA1-independent CEC. By contrast association of immunoglobulins (Igs) on L-HDL-P was a negative determinant of ABCA1-independent efflux particularly in T1DM and likely an important biomarker of HDL dysfunction in this group. In T2DM, association of ApoE (r=0.613) and Vitamin-K dependent protein C (r=0.649) on L-HDL-P were positively associated with ABCA1-dependent CEC, while ApoC-II was a positive determinant of ABCA1-independent efflux (r=0.464). Interestingly, a significant correlation between ApoA-I and ApoA-II on L-HDL-P with ABCA1-independent CEC was evident in both the non-obese (r=0.604 and r=0.630) and obese (r=0.515 and r=0.474) control groups; these associations were completely abrogated in both T1DM (r=0.165 and r=0.199) and T2DM (r=−0.085 and r=0.066). It will be critical to evaluate in future studies whether post-translational modifications unique to the diabetes microenvironment (e.g., glycation) are responsible for this loss of association between ApoA-I and ApoA-II and CEC. These findings stress the importance of developing unique biomarker panels for people with diabetes to capture and manage their CVD risk profile in a more precision medicine-based manner.

Chapter 4 is one of the first studies to disentangle the relationship between HDL proteomic composition and HDL-CEC and furthermore to investigate both the large and small HDL proteome and their subsequent relationship to total CEC, ABCA1-dependent CEC and ABCA1-independent CEC in people with and without T1DM or T2DM. We demonstrate for the first time that there are divergent effects of T1DM and T2DM on HDL-P size, efflux capacity and proteomic composition. We demonstrate that the concentration of L-HDL-P increases in

T1DM, while the concentration of S-HDL-P decreases; by contrast the concentration of S-HDL-P increases significantly with T2DM relative to T1DM. Multiple regression revealed the divergent effects of diabetes status on HDL-CEC, with T1DM status associated with an increase in total and specifically ABCA1-independent CEC, while T2DM status was associated with a decrease in total and specifically ABCA1-dependent CEC when controlling for sex, smoking, age, total cholesterol, TG, HDL-C total HDL-P, HDL size and medications. In our previously publish study we also found that T1DM was associated with an increase in ABCA1-independent CEC when controlling for clinical and biochemical variables [26]. Additionally, He *et al.,* observed an impairment in ABCA1-specific efflux in T2DM, while ABCG1-mediated efflux was unimpeded [27], which match our findings. Our results highlight that HDL-CEC is differentially impaired in T1DM and T2DM and reduced HDL-CEC is a likely contributor to enhanced CVD risk in T2DM. By contrast, HDL protein quality in T1DM is markedly modulated with depletion of complement C3, anti-thrombin-III and pro-thrombin and enrichment with SAA2-4 on L-HDL-P and depletion of LCAT, PON1 and ApoD and enrichment with complement C2 and complement component C9 on S-HDL-P. Collectively these findings highlight a potential shift towards more pro-inflammatory particles in T1DM that may in turn impact on atheroprotective properties beyond HDL-CEC. Another limitation of this study is our focus on HDL-CEC as a biomarker of HDL function. Our proteomics data indicate that measurement of the anti-inflammatory properties of the particles will be critical to also evaluate and is currently being pursued within the laboratory.

Sex-specific differences exist in HDL biology, with men tending to have a less favourable lipoprotein-lipid profile, with more S-HDL-P compared to women [28]. Therefore, combining men and women into a single cohort may result in the loss of unique sex-specific differences. Hence, in the last results chapter, Chapter 5, we examined sex-specific differences in HDL protein composition and function in people with and without T1DM, T2DM and obesity. Our findings support the hypothesis that there are profound differences in HDL biology between the sexes. We found that both women with T1DM and women with T2DM are disadvantaged in terms of HDL-CEC relative to male counterparts. We also report sex-specific differences in the proteins governing HDL-CEC in women versus men that may have important implications for their respective cardiovascular risk profile. In particular, ApoA-I, ApoA-II, ApoC-I, ApoC-III, ApoC-IV, ApoD, ApoE, ApoF and ApoM, were more dominant drivers of CEC in women compared to men, while complement proteins, C3/C5 convertase, C4b-binding protein alpha chain, complement component alpha, beta and gamma chain and complement component 9, were positive drivers of CEC in men compared to women. Of interest, association of Igs on HDL were negatively associated with CEC in men but positively associated in women. Our findings indicate the use of separate biomarkers to predict HDL function in men and women.

Most of our understanding of CVD pathogenesis as well as treatment guidelines derives from experimental and clinical studies conducted on males [29-32]. This myopic approach has led to the deficiencies in care for women with CVD that have been noted [33, 34]. Current clinical CVD risk management strategies are largely undifferentiated between the sexes [35]. Feldman *et al*. reviewed the participation of women and men in clinical trials and research studies between 1966 and 2018. They found that amongst 43,135 published studies and 13,135 clinical trial records, an average of 49% of participants were women [36]. This may seem fair at first glance, however, when the data were sub-categorised by disease, women were underrepresented for diseases they were at higher risk of developing. For example, 51% of individuals with CVD are women but women represented only 39% of research participants into CVD therapeutics.

It is to be assumed that there are dangers in treating women with drugs tested on men at similar dose and strength. In Ireland, the two largest awardees of research grants, Science Foundation Ireland (SFI) and the Irish Research Council (IRC) have not specified sex inclusion criteria in their research strategy for recruiting participants to their respective studies. SFI have a gender strategy focused on attracting researchers into STEM fields but not in requiring recipients of funding to enrol women participants in their research [37]. Furthermore, the IRC does not provide any details on minimum percentages of women and men needed as study participants for grants to be awarded/awarded in the past.

Considering women's underrepresentation in preclinical (cellular and animal models) and clinical research efforts, a broader women's health initiative is needed, integrating sexual and reproductive health with CVD as well as other non-communicable diseases. Sufficient numbers of women should be included in studies of diseases, with a sex-disaggregated approach taken to collecting, analysing and reporting diseases. Given that women are approximately 50% of the population, it seems reasonable, moving forward, that responsible official bodies and journals should mandate that all research (except in extenuating circumstances or in sex-specific conditions) report results by sex. Our results support this type of initiative and indicates that differences in HDL biology may contribute to differences in CVD risk and pathophysiology between men and women.

An important limitation of this thesis is the inability to make causal inference. This thesis presents correlative data which does not infer causation. All studies in this thesis were cross-sectional; therefore, they were not designed to determine the longitudinal effects of metabolic disease on HDL-P function and/or composition and subsequent incidence or progression of CVD. Additionally, it's important to note that the age range of the people studied in this thesis

precedes when active CVD precipitates. Since dyslipidaemia may be present several years before the onset of diabetes, particularly before T2DM, it is hard to predict which of the changes in HDL-P biology are related to the pathognomonic features of disease, and which precede and/or accelerate its progression. Future, follow-up studies over people's lifetimes would be needed to evaluate the longitudinal effects of metabolic disease and sex on HDL-P function and composition, particularly to tease apart HDL-associated proteins causally associated with HDL dysfunction from those that are merely correlative. The HDL-associated proteins we have observed as different throughout the chapters of this thesis may serve as biomarkers of dysfunctional HDL in T1DM or T2DM. However, validating the usefulness of biomarker candidates is a necessity. Further research will be required to unravel HDL-proteins causally associated with HDL dysfunction from those that are merely correlative.

An approach to investigating causality is triangulation, which is used to increase the credibility and validity of research findings [38]. This is where evidence is accumulated from various types of studies for example the relevant types of studies in the field of HDL biology would be animal studies, *in vitro* studies, GWAS, longitudinal studies, intervention studies, observational studies etc. If accumulated evidence from these different methods points to the same conclusions than causality can be assumed/inferred. Central to triangulation is the idea that methods leading to the same results give more confidence to the research findings. While the area of HDL biology has a wide body of literature available, there are still many gaps in our collective knowledge and hence, HDL structure-function relationships still need to be investigated.

In conclusion, this thesis provides evidence for an important role of the HDL proteome in governing HDL-CEC. Our findings demonstrate that HDL functionality and composition are differentially modulated in individuals with T1DM and T2DM and different patterns of proteins are associated with HDL-efflux capacity in these disease states. Additionally, we report sex-specific differences in the proteins governing HDL-CEC in women versus men that may have important implications for their respective cardiovascular risk profile. The findings from this thesis highlight the importance of developing specific protein panels and algorithms for people with and without metabolic disease, and for the sexes separately, to optimally manage CVD risk within these populations. Indeed, within the next decade availability of a standardised high-throughput HDL proteomics assay, along with stratified protein panels and algorithms based on sex and metabolic disease status, may finally realise the full potential of the HDL proteome as the 'pot of gold' and the next frontier in CVD risk management.

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Appendix A

Published paper:

HDL particle size is increased, and HDL-cholesterol efflux is enhanced in type 1 diabetes: a cross sectional study

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ARTICLE

HDL particle size is increased and HDL-cholesterol efflux is enhanced in type 1 diabetes: a cross-sectional study

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Abstract

Aims/hypothesis The prevalence of atherosclerosis is increased in type 1 diabetes despite normal-to-high HDL-cholesterol levels. The cholesterol efflux capacity (CEC) of HDL is a better predictor of cardiovascular events than static HDL-cholesterol. This cross-sectional study addressed the hypothesis that impaired HDL function contributes to enhanced CVD risk within type 1 diabetes.

Methods We compared HDL particle size and concentration (by NMR), total CEC, ATP-binding cassette subfamily A, member 1 (ABCA1)-dependent CEC and ABCA1-independent CEC (by determining [^{3}H]cholesterol efflux from J774-macrophages to ApoB-depleted serum), and carotid intima–media thickness (CIMT) in 100 individuals with type 1 diabetes (37.6 ± 1.2 years; BMI 26.9 ± 0.5 kg/m^2) and 100 non-diabetic participants (37.7 ± 1.1 years; 27.1 ± 0.5 kg/m^2).

Results Compared with non-diabetic participants, total HDL particle concentration was lower (mean ± SD 31.01 ± 8.66 vs 34.33 ± 8.04 μmol/l [mean difference (MD) −3.32 μmol/l]) in participants with type 1 diabetes. However, large HDL particle concentration was greater (9.36 ± 3.98 vs 6.99 ± 4.05 μmol/l [MD +2.37 μmol/l]), resulting in increased mean HDL particle size (9.82 ± 0.57 vs 9.44 ± 0.56 nm [MD +0.38 nm]) ($p < 0.05$ for all). Total CEC (14.57 ± 2.47%CEC/4 h vs 12.26 ± 3.81%CEC/4 h [MD +2.31%CEC/4 h]) was greater in participants with type 1 diabetes relative to non-diabetic participants. Increased HDL particle size was independently associated with increased total CEC; however, following adjustment for this in multivariable analysis, CEC remained greater in participants with type 1 diabetes. Both components of CEC, ABCA1-dependent (6.10 ± 2.41%CEC/4 h vs 5.22 ± 2.57%CEC/4 h [MD +0.88%CEC/4 h]) and ABCA1-independent (8.47 ± 1.79% CEC/4 h vs 7.05 ± 1.76% CEC/4 h [MD +1.42% CEC/4 h]) CEC, were greater in type 1 diabetes but the increase in ABCA1-dependent CEC was less marked and not statistically significant in multivariable analysis. CIMT was increased in participants with type 1 diabetes but in multivariable analysis it was only associated negatively with age and BMI.

Conclusions/interpretation HDL particle size but not HDL-cholesterol level is independently associated with enhanced total CEC. HDL particle size is greater in individuals with type 1 diabetes but even after adjusting for this, total and ABCA1-independent CEC are enhanced in type 1 diabetes. Further studies are needed to understand the mechanisms underlying these effects, and whether they help attenuate progression of atherosclerosis in this high-risk group.

Keywords HDL particle size · HDL-cholesterol efflux capacity · Type 1 diabetes

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Research in context

What is already known about this subject?

- Increased atherosclerotic disease in type 1 diabetes is not fully explained by traditional cardiovascular risk factors
- HDL-cholesterol efflux capacity (CEC) is a better predictor of cardiovascular outcome than HDL-cholesterol levels
- HDL-cholesterol levels are increased in type 1 diabetes but there is some evidence that this increase is not associated with improved HDL function

What is the key question?

- Does HDL CEC differ in individuals with type 1 diabetes compared with similar people without diabetes, and are any observed changes related to HDL particle size?

What are the new findings?

- HDL particle size, but not HDL-cholesterol level, is independently associated with enhanced total CEC. HDL particle size is greater in type 1 diabetes but, even after adjusting for this, total and particularly ATP-binding cassette subfamily A, member 1 (ABCA1)-independent CEC are enhanced in type 1 diabetes

How might this impact on clinical practice in the foreseeable future?

- Measurements of HDL particle size and function might be more useful than HDL-cholesterol levels in evaluating cardiovascular risk in type 1 diabetes

Abbreviations

ABCA1	ATP-binding cassette subfamily A, member 1
ABCG1	ATP-binding cassette subfamily G, member 1
CEC	Cholesterol efflux capacity
CETP	Cholesteryl ester transfer protein
CIMT	Carotid intima–media thickness
JUPITER	Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
MACE	Major atherosclerotic cardiac events
RCT	Reverse cholesterol transport
SAA	Serum amyloid A

Introduction

Despite many improvements in the management of type 1 diabetes, life expectancy for affected individuals remains approximately 12 years less than for the non-diabetic population [1, 2]. This is mostly attributable to an elevated risk of CVD that is not explained by conventional risk factors. In contrast to individuals with type 2 diabetes, lipid profiles in type 1 diabetes are normal or even apparently better than in the general population, with higher HDL-cholesterol, lower LDL-cholesterol and lower triacylglycerol levels [3]. However, these relatively simple lipid measurements potentially mask more subtle lipoprotein abnormalities, including disorders of lipoprotein function, that might contribute to residual atherosclerosis risk in type 1 diabetes.

The importance of a more complete understanding of HDL biology is emphasised by the observation that although HDL-cholesterol levels are inversely associated with CVD [4], increasing HDL-cholesterol levels using cholesteryl ester transfer protein (CETP) inhibitors was found not to result in clinical benefits in high-risk individuals [5, 6]. HDL particles exert numerous atheroprotective functions including antioxidant, anti-inflammatory and cholesterol efflux promoting actions [7]. HDL particles play a critical role in the reverse cholesterol transport (RCT) pathway by promoting efflux of cholesterol from peripheral cells, including lipid-laden foam cells within atherosclerotic lesions, and delivering this acquired lipid back to the liver [8]. Non-esterified cholesterol can be shunted from the liver to the gallbladder via the ABCG5/8 heterodimer [9] or converted into bile acids and shuttled to the gallbladder via ABCB11 [10]. Macrophages efflux cholesterol to smaller HDL particles via the ATP-binding cassette subfamily A, member 1 (ABCA1) transporter while larger particles interact with ATP-binding cassette subfamily G, member 1 (ABCG1) and scavenger receptor class B member 1 (SR-B1) transporters [11]. Evidence that the potentially atheroprotective effects of HDL are clinically relevant is provided by a significant body of literature demonstrating cross-sectional and longitudinal associations between the cholesterol efflux capacity (CEC) of HDL and evidence of both atherosclerosis and cardiovascular events [12–14].

There are theoretical reasons why HDL function in individuals with type 1 diabetes might differ from that in non-diabetic individuals [3]. These include greater particle size,

reduced HDL triacylglycerol content, reduced cholesterol esters in small HDL3 particles, increased susceptibility to oxidation, increases in phospholipid transfer protein (PLTP), CETP and lecithin-cholesterol acyltransferase (LCAT), and changes in the HDL proteome (summarised in detail in [15]). Data from the Pittsburgh Epidemiology of Diabetes Complications Study demonstrate that the usual inverse association between HDL-cholesterol and coronary artery disease risk, although retained in men, is altered in women with type 1 diabetes; the women show little increased protection with concentrations above the range of 50–60 mg/dl (1.3–1.55 mmol/l), suggesting that at least in some individuals, HDL is dysfunctional [16]. Few studies to date have addressed HDL function in type 1 diabetes and none relate aspects of HDL function to early evidence of atherosclerosis.

The aims of this study were to compare total CEC, ABCA1-dependent CEC and ABCA1-independent CEC between individuals with type 1 diabetes and matched control participants to determine whether any observed between-group differences were associated with differences in HDL particle size. We also aimed to determine whether HDL particle size and/or CEC were associated with carotid intima–media thickness (CIMT), a marker of early atherosclerosis.

Methods

Study design and participants This was a cross-sectional study designed to compare HDL particle concentration, HDL particle size and CEC between 100 participants with type 1 diabetes and 100 non-diabetic participants matched for age ±5 years, sex and BMI ±3 kg/m^2.

Participants with type 1 diabetes were contacted either by phone or at the time of their scheduled clinic visit. Inclusion criteria were as follows: type 1 diabetes; duration of diabetes of at least 1 year; age 18–69 years; BMI 18–40 kg/m^2; and HbA_{1c} ≤97 mmol/mol (≤11%). Exclusion criteria were as follows: non-white ethnicity; pregnant or lactating; and recent illness or any chronic illness likely to influence results. Each participant with type 1 diabetes was pair-matched with a healthy volunteer on no medications, recruited from the general population. Inclusion criteria were as follows: non-diabetic; age 18–69 years; and BMI 18–40 kg/m^2. Exclusion criteria were as for participants with type 1 diabetes. Twenty-seven participants with type 1 diabetes and no non-diabetic participants were on statin treatment.

Ethical approval All participants gave written informed consent to the study, which was approved by the Research Ethics Committee of the Adelaide and Meath Hospital and St James' Hospital (Dublin, Ireland).

Study protocol and anthropometric data All participants were studied after they had fasted for 12 h, having avoided excessive exercise and alcohol for the previous 24 h. Height was measured with a Harpenden stadiometer. Weight was measured in a hospital gown. Data relating to previous medical history, medication use and smoking status were recorded. Alcohol consumption was recorded in all participants with type 1 diabetes and in 80 of 100 non-diabetic participants.

Laboratory methods Plasma levels of total cholesterol, triacylglycerols and HDL-cholesterol were measured by an enzymatic calorimetric method on the Roche P Module (Roche, Mannheim, Germany). Additional samples were centrifuged at 1500 *g* for 15 min at 4°C, and plasma and serum were stored at −80°C until the end of the study.

CEC J774 murine macrophages, derived from BALB/c mice (European Collection of Authenticated Cell Cultures), were seeded (7×10^5 cells/ml) onto 96-well plates for 24 h, and cultured at 37°C. Cells were subsequently labelled with [^{3}H]cholesterol (1 μCi/ml) for 24 h before being equilibrated for 18 h in DMEM containing 0.2% BSA with or without cAMP (0.3 mmol/l) to drive ABCA1 expression. J774 macrophages do not express ABCA1 basally, and stimulation with cAMP specifically drives ABCA1 protein expression. ApoB-containing lipoproteins were removed from serum by polyethylene glycol precipitation, leaving an HDL-enriched supernatant fraction. Ex vivo efflux from labelled macrophages to 2.8% HDL-enriched supernatant fraction in minimal essential media (MEM) was measured over 4 h. Percentage efflux was determined by liquid scintillation counting. The difference in efflux to HDL from cells stimulated in the presence and absence of cAMP was taken to represent ABCA1-dependent CEC while ABCA1-independent CEC was calculated from unstimulated cells.

NMR HDL particle concentration and size were measured in serum samples using 400 MHZ NMR spectroscopy (LipoScience, Raleigh, NC, USA). Each lipoprotein subclass emits a distinctive lipid methyl group NMR signal and the amplitudes of the measured signals are directly proportional to the number of subclass particles, which in turn provides the estimated mol/l concentration for the three HDL particle subclasses (large 8.9–13 nm, medium 8.3–8.8 nm, small 7.3–8.2 nm) [17]. Weighted-average HDL particle sizes in nanometres were calculated from the subclass levels, and the diameters were assigned to each subclass.

CIMT CIMT was measured as previously described [18] in all participants using a single ultrasonographic machine (Sonosite Micromaxx Ultrasound System, Sonosite, Japan). Measurements were taken from each common carotid artery,

within 1 cm of the carotid bulb, with three views of each artery measured, and averaged to form a composite measurement.

Statistical analysis Results in tables are presented as mean ± SD unless otherwise specified. Data in graphs are presented as mean ± SD. Data were tested for normality using the Shapiro–Wilk test, as well as inspected visually using histograms. Statistical significance for categorical variables was calculated using the χ^2 test. For continuous variables, the paired Student's *t* test was used to assess statistical significance. Correlations were evaluated using the Pearson correlation or Spearman test as appropriate. Generalised estimating equations with robust Huber–White (sandwich) SE estimation was used to examine the joint effect of all predictors while accounting for the non-independence of matched pairs. IBM SPSS Statistics (Version 24.0. Armonk, NY) and GraphPad Prism (Version 8.4.3. San Diego, CA) software were used for data analysis. Statistical significance is presented as $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results

Clinical and biochemical characteristics of 100 participants with type 1 diabetes and 100 healthy non-diabetic participants are shown in Table 1. There were no significant differences in total cholesterol or triacylglycerol levels between type 1 diabetes and control groups. LDL-cholesterol was lower, HDL-cholesterol and HbA_{1c} were higher, and CIMT was greater in participants with type 1 diabetes than in non-diabetic participants (Table 1, Fig. 1). Mean alcohol consumption was greater in the non-diabetic participants, although the difference did not reach statistical significance.

HDL particle size and subclass distribution Total HDL particle concentration was significantly lower in participants with type 1 diabetes (Fig. 1a) than in non-diabetic participants despite HDL-cholesterol concentrations being higher (Table 1). This reduction was specifically evident in women (mean ± SD for type 1 diabetes vs non-diabetic group: 28.42 ± 9.64 vs 34.91 ± 9.22 μmol/l [mean difference −6.49 μmol/l]) with no effect evident in men (34.79 ± 5.07 vs 33.77 ± 5.99 μmol/l [mean difference 1.02 μmol/l]) (Fig. 1a). Mean HDL particle size was greater in participants with type 1 diabetes than in non-diabetic participants, with similar effects evident in men and women (Fig. 1b). Consistent with an increase in mean HDL particle size, the concentration of large HDL particles was increased in participants with type 1 diabetes, with similar patterns evident in men (7.09 ± 3.34 vs 4.56 ± 2.15 μmol/l [mean difference +2.53 μmol/l]) and women (10.92 ± 3.64 vs 8.68 ± 4.25 μmol/l [mean difference +2.24 μmol/l]) (Fig. 1c). By contrast, the concentration of medium and small HDL particles was significantly reduced in participants with type 1 diabetes relative to non-diabetic participants (Fig. 1d,e). These effects were particularly pronounced in women: mean ± SD HDL particle concentration for type 1 diabetes vs non-diabetic groups was 10.08 ± 6.91 vs 13.64 ± 7.77 μmol/l (mean difference −3.56 μmol/l, $p > 0.05$) for medium HDL particles and 7.44 ± 5.77 vs 12.53 ± 7.03 μmol/l (mean difference −5.09 μmol/l, $p < 0.001$) for small HDL particles. A non-

Table 1 Baseline characteristics of type 1 diabetes and non-diabetic cohorts

Characteristic	T1DM (*n*=100)	Non-diabetic (*n*=100)	Mean difference	95% CI	*p* value
Sex, *n* male/*n* female	40/60	40/60	NA	NA	1.0
Age, years	37.6 ± 11.9	37.7 ± 11.0	−0.1	−0.6, 0.5	0.8
BMI, kg/m^2	26.9 ± 4.9	27.1 ± 5.0	−0.2	−0.4, 0.1	0.13
Smoker, %	20	18	N/A	N/A	0.68
Alcohol intake, units/week	2 (0–8)	6 (0–12)	N/A	−4.9, −0.1	0.04*
Systolic BP, mmHg	128 ± 16	127 ± 15	1	−3, 5	0.58
Diastolic BP, mmHg	77 ± 9	82 ± 11	−6	−8, −3	<0.0001***
CIMT, mm	0.590 ± 0.084	0.556 ± 0.071	0.034	0.003, 0.047	0.03*
HbA_{1c}, mmol/mol	70 ± 16	35 ± 3	35	32, 39	<0.0001***
HbA_{1c}, %	8.5 ± 1.5	5.3 ± 0.3	3.2	2.9, 3.6	<0.0001***
Total cholesterol concentration, mmol/l	4.5 ± 0.9	4.7 ± 0.9	−0.2	−0.4, 0.1	0.32
Triacylglycerol concentration, mmol/l	0.9 (0.7–1.3)	0.9 (0.7–1.3)	N/A	−0.1, 0.3	0.37
LDL-cholesterol concentration, mmol/l	2.3 ± 0.9	2.7 ± 0.8	−0.4	−0.6, −0.1	0.004**
HDL-cholesterol concentration, mmol/l	1.6 ± 0.4	1.5 ± 0.4	0.1	0.1, 0.3	0.002**

Data are displayed as mean±SD, median (IQR) or %

*p<0.05, **p<0.01 and ***p<0.001 for type 1 diabetes vs non-diabetic cohort

T1DM, type 1 diabetes

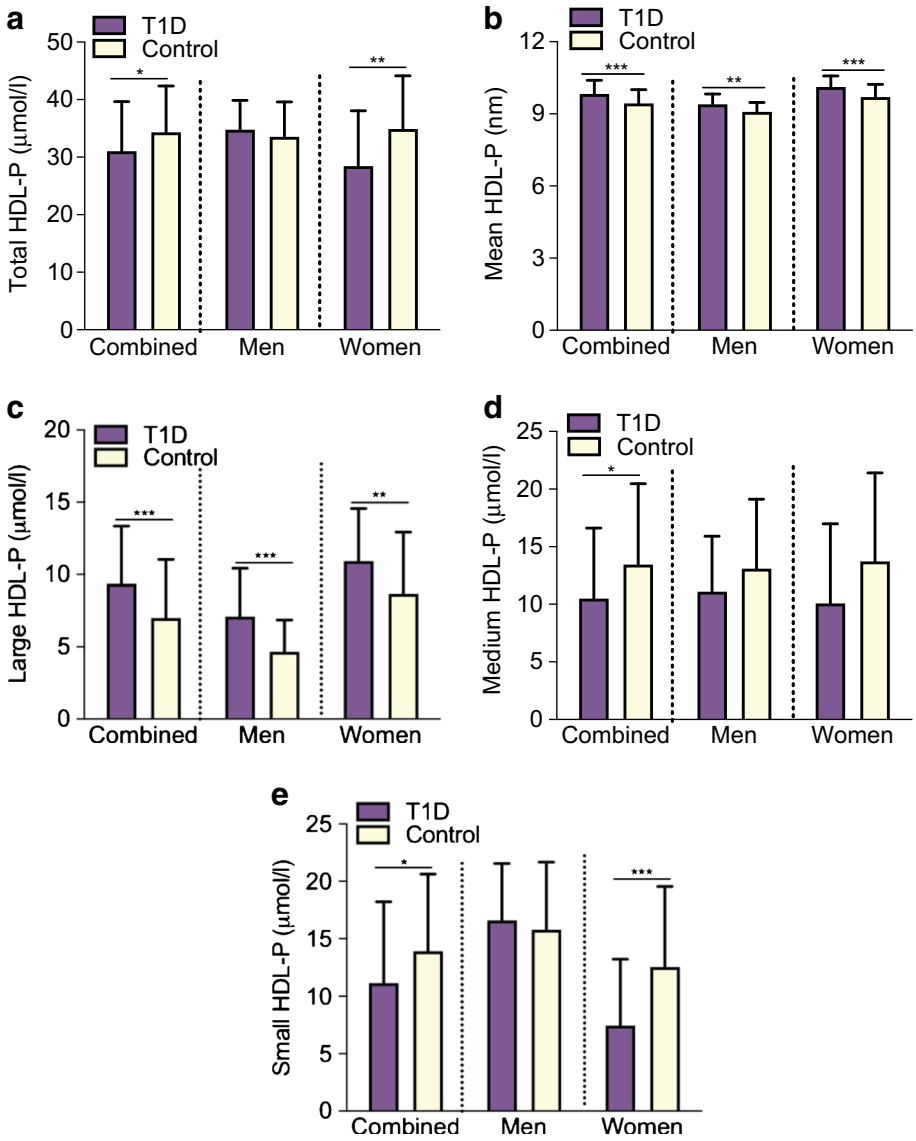

Fig. 1 NMR analysis of HDL particles in individuals with type 1 diabetes and non-diabetic participants (control), showing results for total cohort combined (n=100) and separately for men (n=40) and women (n=60). Bar charts show total HDL particle concentration (**a**), mean HDL particle diameter (**b**), large HDL particle concentration (**c**), medium HDL particle concentration (**d**) and small HDL particle concentration (**e**). Data are presented as mean ± SD. *p<0.05, **p<0.01 ***p<0.001 for type 1 diabetes vs non-diabetic control. HDL-P, HDL particle; T1D, type 1 diabetes

significant reduction in medium HDL particle concentration (11.1 ± 4.8 vs 13.11 ± 6.12 μmol/l [mean difference −2.01 μmol/l]), and a non-significant increase in small HDL particle concentration (16.6 ± 4.95 vs 16.1 ± 5.66 μmol/l [mean difference +0.5 μmol/l]) was evident in men with type 1 diabetes relative to non-diabetic participants (Fig. 1d,e).

HDL CEC Total CEC was significantly greater in participants with type 1 diabetes than in non-diabetic participants (Fig. 2a, Table 2). A significant increase in both ABCA1-dependent and ABCA1-independent efflux was evident in participants with type 1 diabetes relative to non-diabetic participants (Fig. 2b,c). Interestingly, total CEC and ABCA1-independent CEC were increased to a greater extent relative to non-diabetic participants in men (14.48 ± 2.51%CEC/4 h vs 11.02 ± 4.03%CEC/4 h [mean difference +3.46%CEC/4 h] and 9.16 ± 1.65%CEC/4 h vs 6.43 ± 2.06%CEC/4 h [mean difference +2.73%CEC/4 h], respectively) than in women (14.63 ± 2.46%CEC/4 h vs 13.16 ± 3.46%CEC/4 h [mean difference +1.47%CEC/4 h] and 8.01 ± 1.74%CEC/4 h vs 7.44 ± 1.42%CEC/4 h [mean difference +0.57%CEC/4 h], respectively) (Fig. 2a,c). In parallel, CIMT burden was a significantly greater in men with type 1 diabetes relative to non-diabetic men (0.618 ± 0.09 vs 0.566 ± 0.07 mm [mean difference +0.052 mm], p < 0.01) with no significant difference evident in women (Fig. 2d).

Effect of alcohol consumption To ensure the observed differences were not due to the finding that mean alcohol consumption was (non-significantly) greater in the non-diabetic individuals, comparisons were repeated in participants with an alcohol intake ≤10 units/week (79/100 participants with type 1 diabetes; 59/80 non-diabetic participants). There was no difference in findings (mean ± SD values for participants with

Table 2 CEC and lipoprotein particle analysis in type 1 diabetes and non-diabetic cohorts

Variable	T1DM (n=100)	Non-diabetic (n=100)	Mean difference	95% CI
Total CEC, %CEC/4 h	14.57 ± 2.47	12.26 ± 3.81	+2.31	1.36, 3.35 ***
ABCA1-dependent CEC, %CEC/4 h	6.10 ± 2.41	5.22 ± 2.57	+0.88	0.19, 1.66 *
ABCA1-independent CEC, %CEC/4 h	8.47 ± 1.79	7.05 ± 1.76	+1.42	0.90, 1.97 ***
Mean HDL-P size, nm	9.82 ± 0.57	9.44 ± 0.56	+0.38	0.24, 0.49 ***
Total HDL-P concentration, μmol/l	31.01 ± 8.66	34.33 ± 8.04	−3.32	−5.57, −0.29 *
Large HDL-P concentration, μmol/l	9.36 ± 3.98	6.99 ± 4.05	2.37	1.48, 3.41 ***
Medium HDL-P concentration, μmol/l	10.49 ± 6.13	13.45 ± 7.02	−2.96	−5.01, −0.48 *
Small HDL-P concentration, μmol/l	11.16 ± 7.06	13.90 ± 6.7	−2.74	−4.63, −0.62 *

Data are displayed as mean±SD

*p<0.05 and ***p<0.001 for type 1 diabetes vs non-diabetic cohort

HDL-P, HDL particle; T1DM, type 1 diabetes

type 1 diabetes vs non-diabetic participants: HDL particle size 9.84 ± 0.59 vs 9.43 ± 0.55 nm, $p < 0.0005$; total CEC 14.52 ± 2.49%CEC/4 h vs 12.32 ± 4.01%CEC/4 h $p < 0.0005$; ABCA1-dependent CEC 6.19 ± 2.47%CEC/4 h vs 5.24 ± 2.70%CEC/4 h, $p < 0.05$; ABCA1-independent CEC 8.32 ± 1.84%CEC/4 h vs 7.07 ± 1.83%CEC/4 h, $p < 0.0005$).

Univariable and multivariable associations In univariable analysis, total CEC was positively associated with type 1 diabetes status (greater in type 1 diabetes vs no diabetes), total cholesterol concentration and HDL particle size, and negatively associated with age (Table 3). In multivariable analysis, total CEC was positively associated with type 1 diabetes status, total cholesterol concentration, HDL particle size and statin treatment (greater with vs without statin treatment), and negatively associated with age (Table 3).

In univariable analysis, ABCA1-dependent CEC was positively associated with total cholesterol and triacylglycerol concentration (Table 4). In multivariable analysis, ABCA1-dependent CEC was positively associated with total cholesterol concentration and negatively associated with age (Table 4).

In both univariable and multivariable analysis, ABCA1-independent CEC was positively associated with type 1 diabetes status, HDL particle concentration and statin treatment (Table 5).

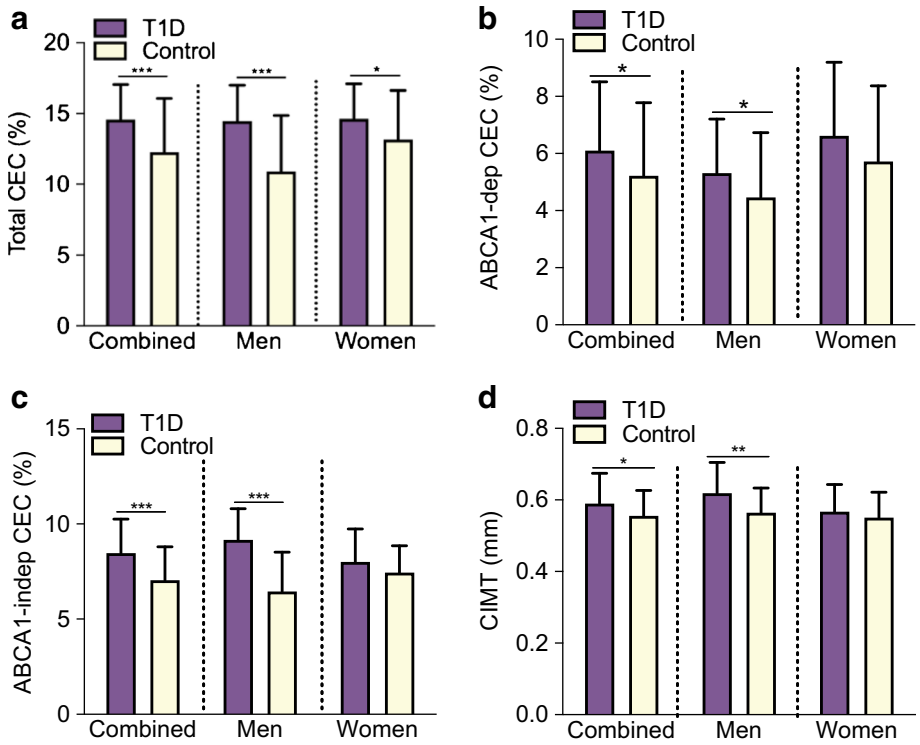

Fig. 2 HDL CEC and CIMT measurements in participants with type 1 diabetes and non-diabetic participants (control), showing results for total cohort combined (n=100) and separately for men (n=40) and women (n=60). Bar charts show total CEC (**a**), ABCA1-dependent CEC (**b**), ABCA1-independent CEC (**c**) and CIMT measurements (**d**). Data are presented as mean ± SD. Statistical significance is presented as *p<0.05, **p<0.01 and ***p<0.001 for type 1 diabetes vs non-diabetic control. T1D, type 1 diabetes

Table 3 Univariable and multivariable association of clinical and biochemical variables with HDL CEC

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	−0.061	−0.116, −0.007	−0.061	−0.114, −0.008
Sex (1 male, 2 female)	0.540	−0.696, 1.776	0.624	−1.882, 0.634
BMI (kg/m^2)	0.006	−0.094, 0.106	0.002	−0.082, 0.085
Type 1 diabetes (1 no, 2 yes)	1.507	0.318, 2.695	1.465	0.265, 2.666
Total cholesterol (mmol/l)	0.885	0.195, 1.576	0.870	0.195, 1.545
Triacylglycerols (mmol/l)	0.586	−0.249, 1.421	0.629	−0.351, 1.608
HDL-cholesterol (mmol/l)	0.804	−1.581, 3.189	0.600	−2.206, 3.406
HDL-P concentration (μmol/l)	0.055	−0.033, 0.144	0.050	−0.039, 0.138
HDL-P size (nm)	2.426	0.442, 4.409	2.173	0.183, 4.163
Large HDL-P concentration (μmol/l)	−0.189	−0.477, 0.098	−0.142	−0.420, 0.136
Statin treatment (1 no, 2 yes)	1.988	−0.012, 3.988	2.093	0.625, 3.561

β coefficient was derived from linear regression and generalised estimating equation

HDL-P, HDL particle

In univariable analysis, CIMT was positively associated with ABCA1-dependent CEC and ABCA1-independent CEC and negatively associated with age and BMI (data not shown). In multivariable analysis, CIMT was negatively associated with age and BMI.

Discussion

We have demonstrated reduced total HDL particle concentration in type 1 diabetes, but greater large HDL particle concentration, resulting in greater mean HDL particle size. Increased HDL particle size was independently associated with increased total CEC; however, even following adjustment for increased HDL-cholesterol and HDL particle size in multivariable analysis, CEC remained greater in participants with type 1 diabetes compared with closely matched participants without diabetes. Both components of CEC, ABCA1-dependent and ABCA1-independent CEC, were greater in type 1 diabetes but the increase in ABCA1-dependent CEC was less marked and not statistically significant in multivariable analysis.

This is the first study in type 1 diabetes to determine ABCA1-dependent and ABCA1-independent contributions to CEC, and the first to address associations between CEC, HDL particle size and HDL particle concentration, and

Table 4 Univariable and multivariable association of clinical and biochemical variables with ABCA1-dependent CEC in participants with type 1 diabetes and participants without diabetes

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	−0.040	−0.081, 0.001	−0.041	−0.077, −0.004
Sex (1 male, 2 female)	0.731	−0.201, 1.664	0.827	−1.745, 0.092
BMI (kg/m^2)	−0.011	−0.086, 0.65	−0.017	−0.086, 0.053
Type 1 diabetes (1 no, 2 yes)	0.073	−0.824, 0.969	0.078	−0.965, 0.810
Total cholesterol concentration (mmol/l)	0.700	0.179, 1.221	0.686	0.151, 1.222
Triacylglycerol concentration (mmol/l)	0.698	0.068, 1.328	0.739	−0.036, 1.515
HDL-cholesterol concentration (mmol/l)	0.572	−1.227, 2.371	0.432	−1.974, 2.838
HDL-P concentration (μmol/l)	−0.055	−0.121, 0.012	−0.056	−0.125, 0.012
HDL-P size (nm)	1.447	−0.049, 2.944	1.349	−0.184, 2.882
Large HDL-P concentration (μmol/l)	−0.119	−0.336, 0.097	−0.108	−0.336, 0.121
Statin treatment (1 no, 2 yes)	0.887	−0.622, 2.396	0.973	−2.086, 0.140

β coefficient derived from linear regression and generalised estimating equation

HDL-P, HDL particle

Table 5 Univariable and multivariable association of clinical and biochemical variables with ABCA1-independent CEC in participants with type 1 diabetes and participants without diabetes

Variable	Univariable model		Multivariable model	
	β coefficient	95% CI	β coefficient	95% CI
Age (years)	−0.021	−0.049, 0.007	−0.021	−0.046, 0.004
Sex (1 male, 2 female)	−0.191	−0.823, 0.441	−0.198	−0.384, 0.781
BMI (kg/m^2)	0.017	−0.035, 0.068	0.017	−0.026, 0.061
Type 1 diabetes (1 no, 2 yes)	1.434	0.827, 2.041	1.420	0.835, 2.004
Total cholesterol concentration (mmol/l)	0.185	−0.168, 0.007	0.186	−0.155, 0.527
Triacylglycerol concentration (mmol/l)	−0.112	−0.539, 0.314	−0.115	−0.529, 0.299
HDL-cholesterol concentration (mmol/l)	0.233	−0.986, 1.452	0.215	−1.001, 1.431
HDL-P concentration (μmol/l)	0.110	0.065, 0.155	0.109	0.069, 0.149
HDL-P size (nm)	0.975	−0.039, 1.989	0.927	−0.046, 1.899
Large HDL-P concentration (μmol/l)	−0.070	−0.217, 2.041	−0.058	−0.202, 0.086
Statin treatment (1 no, 2 yes)	1.102	0.079, 2.124	1.103	0.213, 1.993

β coefficient was derived from linear regression and generalised estimating equation

HDL-P, HDL particle

evidence of early atherosclerosis (CIMT). The study includes the following strengths: (1) comparison between a cohort of 100 type 1 diabetic participants and carefully matched non-diabetic participants; (2) profiling of HDL particle size by NMR and corresponding measurement of particle CEC; and (3) measurement of CIMT, an established marker of early atherosclerosis. Limitations include the fact that this was a cross-sectional study and was not designed to determine the longitudinal effect of the measured variables on progression of atherosclerosis and major atherosclerotic cardiac events (MACE). Furthermore, the study was performed within a population of white ethnicity; it is likely that the variables tested will be influenced by ethnicity, which should be an important consideration for future studies. Finally measures of HDL CEC do not capture information regarding the influence of type 1 diabetes on macrophage/cellular efflux capacity; Daffu et al [19] showed previously that murine diabetic macrophages exhibit impaired ABCG1-mediated efflux in an AGE receptor-dependent mechanism while Mauldin et al [20] similarly demonstrated that monocyte-derived macrophages from individuals with type 2 diabetes exhibit reduced CEC. To date, little is known about macrophage efflux capacity in type 1 diabetes.

Most of the studies on HDL-cholesterol levels in type 1 diabetes reported a significant increase in levels compared with non-diabetic control populations (summarised in [15]). Previous smaller studies investigating HDL function in type 1 diabetes have reported conflicting findings. De Vries et al demonstrated increased total CEC in 14 individuals with type 1 diabetes compared with healthy controls [21]. In contrast, Manjunatha et al demonstrated reduced HDL CEC in individuals with type 1 diabetes who had poor glycaemic control and good glycaemic control (15 in each group), relative to matched non-diabetic counterparts [22]; notably differences in CEC were small and plasma HDL-cholesterol concentration was equivalent across groups. The current study confirms that CEC is increased in type 1 diabetes independent of HDL-cholesterol, and that this increase is contributed to particularly by ABCA1-independent CEC.

We demonstrated sex-specific differences in HDL biology in type 1 diabetes with preservation of HDL particle concentration and increased ABCA1-independent efflux evident in men but not in women relative to non-diabetic participants. In contrast, there was a profound reduction in the concentration of small HDL particles in women with type 1 diabetes compared with non-diabetic women, with no effect evident in men. Notably, however, division of type 1 diabetes participants by sex resulted in relatively small cohorts of 40 men and 60 women; these observations will require verification and further analysis in larger type 1 diabetes populations.

The observed increase in CEC in type 1 diabetes occurred in parallel with increased HDL-cholesterol concentration, HDL particle size and large HDL particle concentration. Although not previously demonstrated in populations with type 1 diabetes, the association between larger HDL particles and total CEC has been reported in older adults [23] and perimenopausal women [24]. In the current study, consistent with increased particle size, the increase in total CEC was predominantly attributable to increased ABCA1-independent CEC [11], with a lesser increase in ABCA1-dependent CEC. Interestingly, despite the reduction in concentration of small- and medium-sized HDL particles in type 1 diabetes, ABCA1-dependent CEC was enhanced relative to non-diabetic participants. ABCA1-dependent efflux typically correlates with

lipid-poor pre-β-1 HDL and not with large HDL particles [25] and thus these findings were unexpected; they indicate that the larger-sized HDL particles in type 1 diabetes might facilitate greater storage of non-esterified cholesterol, causing this unexpected change in association between ABCA1-dependent efflux and particle size. It is also important to note that NMR is limited in its ability to detect lipid-poor pre-β-1 HDL [26] as it specifically measures cholesterol on lipoprotein particles. These lipid-poor pre-β-1 HDL particles are the primary acceptor of ABCA1-dependent CEC [27] and thus we potentially did not capture the true association between ABCA1-dependent CEC and small HDL particles in the current study.

We previously demonstrated increased association of the acute-phase inflammatory protein serum amyloid A (SAA) on HDL2 and HDL3 subfractions in type 1 diabetes [28]. Enrichment of HDL with SAA has been reported to reduce CEC [29, 30], particularly in an acute-phase setting [31, 32], and impair the anti-inflammatory properties of the HDL particles [33]. The degree of enrichment of HDL with SAA during the acute-phase response (~1000-fold) [34], however, is considerably higher than that seen in type 1 diabetes (~1.7 fold) and therefore differences observed in type 1 diabetes might not be clinically relevant.

The observed increase in HDL particle size in type 1 diabetes has possible important implications for RCT through the lymphatic vasculature [35]. The level and particle size of HDL within the interstitial fluid that bathes peripheral cells is arguably more important for RCT than the size of particles within serum. Approximately 50% of all ApoA-I circulates within the interstitial fluid of peripheral organs [36], where pre-β particles predominate and CETP activity is low relative to plasma [36]. Recent findings by Apro et al have demonstrated that the CEC of HDL derived from interstitial fluid was significantly lower than that derived from plasma [37]. Furthermore, HDL CEC was significantly reduced in individuals with type 2 diabetes relative to healthy controls and this effect was more pronounced in HDL from interstitial fluid compared with plasma [37]. A limitation of the current study is that we have specifically measured serum CEC and not interstitial fluid CEC. Thus, while serum CEC is enhanced in type 1 diabetes, the shift towards larger particle size might in fact reduce access to the interstitial fluid and reduce this important route of RCT. In particular, the remarkable reduction in small HDL particle number in women may lead to loss of cholesterol flux through the lymphatic RCT pathway. This requires further investigation as it potentially contributes to residual cardiovascular risk in individuals with type 1 diabetes.

The current study demonstrates both potentially protective (increased HDL particle size and CEC) and harmful (reduced HDL particle number) effects of type 1 diabetes on HDL biology. Similar to CEC, measuring the number of HDL particles by NMR has been shown in numerous trials (including the Veterans Affairs High-Density Lipoprotein Intervention Trial [VA-HIT] and the primary prevention Multiple Risk Factor Intervention Trial [MRFIT]) to be a better predictor of future cardiovascular events than circulating HDL-cholesterol or ApoA-I levels [38, 39] (reviewed in reference [40]). Recent subanalysis of findings from Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) has suggested that NMR-measured HDL particle number is superior to CEC in predicting CVD events [41]. Levels of large HDL particle subpopulations, as determined by NMR, are inversely associated with CVD risk while levels of small HDL particles are often positively correlated with risk [40, 42]. Circulating levels of large α-1 HDL, as determined by 2D gel electrophoresis, are also associated with reduced cardiovascular risk while levels of small HDL particles are elevated in individuals with CHD [43–45]. The Epidemiology of Diabetes Complications (EDC) study demonstrated prospectively that HDL particle size could predict the development of cardiovascular events in type 1 diabetes [46]. Similar to our findings, Colhoun et al demonstrated more large HDL particles and fewer small HDL particles in type 1 diabetes, and greater mean HDL particle size [47]; however, they did not find a relationship between particle size and computer tomography-defined coronary artery calcification and in the current study we did not demonstrate associations between any components of HDL structure and function and CIMT.

We observed a positive correlation between both ABCA1-dependent and ABCA1-independent CEC and CIMT, although these effects did not persist in multivariable analysis. While this was unexpected it is noteworthy that the study participants were relatively young (mean age 37 years) and predominantly female (60%) with low lesion burden. Furthermore, the association between CEC and CVD is controversial. The cornerstone study by Khera et al demonstrated an association between HDL CEC and CVD burden in an older population (mean age 57 ± 9 years) with angiographically confirmed CVD [12]. Khera et al have more recently demonstrated superiority of measuring HDL particle number compared with HDL CEC as a predictor of incident CVD, finding no association between baseline CEC and incident CVD within JUPITER [41]. However, apparently contrary to these findings, Li et al demonstrated a positive association between CEC and cardiovascular events in non-diabetic individuals (1.66-fold increase in MACE), findings that remained similar (1.85-fold increase) after adjustment for traditional cardiovascular risk factors [48]. Furthermore, in the Multiethnic Study of Atherosclerosis [14], CEC was inversely associated with incident coronary artery disease but positively associated with carotid plaque progression. Taken together, these findings suggest significant complexity in the

association between CEC and progression of atherosclerosis at different sites. Larger studies including type 1 diabetes participants only are needed to clarify whether and to what extent changes in both particle size and CEC influence atherosclerosis risk in this condition.

An important consideration in future studies will be investigation of the effects of statin treatment and alcohol intake on HDL structure and function in diabetes. Although statin treatment was independently and positively associated with CEC in multivariable analysis, it is difficult to separate this apparent effect from the fact that statins were predominantly used in older male participants, and only in the type 1 diabetes group. Alcohol intake was greater in non-diabetic participants and statistically significant correlations were identified between alcohol intake and certain components of CEC. Limiting analysis, however, to participants from both groups with weekly alcohol intake not greater than ten units did not change our findings. Future studies in larger cohorts will help explore potential contributions of statins and alcohol to HDL structure and function in diabetes.

In conclusion, we have demonstrated potentially important differences in CEC, particularly its ABCA1-independent component, in type 1 diabetes. These differences remain statistically significant following adjustment for differences in HDL-cholesterol concentration and HDL particle size and concentration. Future studies are needed to determine the mechanism underlying these differences and their role in the atherosclerotic complications of type 1 diabetes.

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Contribution statement MOA co-designed the study, recruited the participants, measured CIMT, analysed clinical data and prepared the first draft of paper. FCM and JG co-designed the study: JG managed the clinical recruitment and CIMT analysis; and FCM led the laboratory-based analysis of HDL. REB performed the HDL function assays, analysed data, prepared figures and co-wrote the first draft of the paper. WG, AP, AG, IF, MS, KSA, AM and KM were involved in study design, recruitment of participants into the study, clinical measurements of CIMT and reviewed and edited the final manuscript. GB collaborated on study design, sample analysis, data acquisition and reviewed the manuscript. RS planned the re-analysis of data for the revised manuscript, oversaw data analysis and presentation, and description of the new statistical methods and results throughout the text, and helped draft the final manuscript. FCM and JG co-edited the final paper and all authors approved the final draft. JG is responsible for the integrity of the work as a whole.

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