THE IMPACT OF ETHNICITY ON LIPOPROTEINS IN CORONARY ARTERY DISEASE

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Dedicated to;

My wife, Sonia and my children, Jayen & Bella.

Abstract

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Coronary artery disease (CAD) is a leading cause of mortality in the UK, with individuals of South Asian origin at intensified risk, owing to a higher prevalence of diabetes, culminating in an atherogenic phenotype. The epidemiological evidence supporting a protective role for high density lipoprotein-cholesterol in CAD, has failed to be realised in the ILLUMINATE/AIM-high trials. The paradigm has shifted towards an appreciation of the quality of the lipoprotein particles, rather than arbitrary levels. Lipoproteins contain unique protein cargoes, mediating roles in coagulation, redox and inflammation. The objective of this study was to explore differences in the low abundant protein cargo of lipoproteins between South Asian and Caucasian patients with CAD, to further understand the differential risk.

A novel lipoaffinity resin was explored and optimised for efficient and reliable lipoprotein pull-down from plasma. Samples were analysed using a label-free bottom-up proteomic approach on an ion-mobility enabled mass spectrometer. Statins remain the cornerstone for prevention of CAD. In a sub-study, statin therapy was associated with the modulation of proteins concerned with the cytoskeletal architecture, cell proliferation and inflammation, in patients with hypercholesterolemia. The controversial link between statin use and new onset diabetes may be explained a depletion of adipsin, a potent insulin secretagogue. In a further sub-study, lipoproteins of CAD patients were enriched with pro-inflammatory mediators compared with age and sex matched controls. A model comprising of 7 proteins accurately predicted CAD status. In the discovery study, lipoproteins of South Asians were enriched with pro-thrombotic and pro-inflammatory mediators, compared with sex-matched Caucasians with stable CAD. Plasma carboxypeptidase B2 was significantly higher in South Asians compared to Caucasians with CAD in verification studies, contributing to impaired fibrinolysis and potentially to their excess risk.

The lipoproteomic cargo undergoes subtle changes in CAD, with various dysregulated proteins independently influenced by ethnicity.
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Research Publications & Presentations

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List of abbreviations

4S. Scandinavian Simvastatin Survival Study
AAG. Alpha 1 Acid Glycoprotein
ABCA1. ATP Binding Cassette A1 receptor
ACE. Angiotensin Converting Enzyme
ACN. Acetonitrile
ACS. Acute Coronary Syndrome
ACT. Alpha-1-anti-Chymotrypsin
ADH. Alcohol Dehydrogenase
AGC. Automatic Gain Control
AGEs. Advanced Glycation End Products
AmBic. Ammonium Bicarbonate
apo. Apolipoprotein

BASP1. Brain Acid Soluble Protein 1
BCA. Bicinchoninic Acid
BMI. Body Mass Index
BSA. Bovine Serum Albumin

CABG. Coronary Artery Bypass Graft
CAC. Coronary Artery Calcification
CAD. Coronary Artery Disease
CARE. Cholesterol And Recurrent Events trial
CETP. Cholesterol Ester Transfer Protein
CKD. Chronic Kidney Disease
CMR. Cardiac Magnetic Resonance
CRP. C-Reactive Protein
CTO. Chronic Total Occlusions
CV. Coefficient of Variation

DAF. Decay Accelerating Factor
DIPE. Di-Isopropyl Ether
DTT. Dithiothreitol

ESI. Electrospray Ionisation

FA. Formic Acid
FDR. False Discovery Rate
FTICR. Fourier Transform Ion Cyclotron Resonance

GXP. Glutathione Peroxidase

HDL. High Density Lipoprotein
HCD. Higher-energy Collisional Dissociation
HPLC. High Pressure Liquid Chromatography
HUVEC. Human Umbilical Vein Endothelial Cells

IAA. Iodoacetamide
IMS. Ion Mobility Spectrometry
ITIH4. Inter alpha Trypsin Inhibitor Heavy chain H4

LAD. Left Anterior Descending
LCAT. Lecithin-Cholesterol Acyltransferase
LDL. Low Density Lipoprotein
LRA. Lipid Removal Agent

m/z. mass/charge
MALDI. Matrix Assisted Laser Desorption Ionisation
MARS. Multiple Affinity Removal System
MASP. Mannan binding lectin Serine Protease
miRNA. microRNA
MMP-9. Matrix Metallopeptidase-9
MS. Mass Spectrometer

NP40. Nonidet P40

PAF. Platelet Activating Factor
PAH-AH. Platelet Activating Factor Acetylhydrolase
PAI-1. Plasminogen Activating Inhibitor 1, Plasminogen Activator Inhibitor-1
PBS. Phosphate Buffered Solution
PCI. Percutaneous Coronary Intervention
PON-1. Paraoxonase-1
PPARα. Peroxisome Proliferator-Activated Receptor
PROVE-IT TIMI 22. Pravastatin or Atorvastatin Evaluation and Infection Therapy Thrombolysis In Myocardial Infarction 22

QC. Quality Control

RAGE. Receptor for Advanced Glycation End products
ROC. Receiver Operating Characteristic

SDC. Sodium Deoxycholate
SDS. Sodium Dodecyl Sulphate
SNP. Single Nucleotide Polymorphisms
SPE. Solid Phase Extraction
SRB1. Scavenger Receptor B1
SREBP. Sterol Response Element Binding Factor Protein
STRING. Search Tool for the Retrieval of Interacting Genes/proteins

TAFI. Thrombin Activatable Fibrinoylsis Inhibitor
TBP. Tributylphospine
TFA. Trifluoroacetic Acid
TFE. Trifluoroethanol
TOF. Time Of Flight
TQ. Triple Quadrupole
TSP-1. Thrombospondin-1
TULP-2. Tubby related Protein 2
TWIMS. Travelling Wave Ion Mobility Spectrometry

UK. United Kingdom

VLDL. Very Low Density Lipoprotein

WOSCOPS. West of Scotland Coronary Prevention Study

Chapter 1

1 Coronary artery disease

1.1 Introduction

Coronary artery disease (CAD) is due to the formation and progression of complex atherosclerotic lesions in the coronary arteries which disrupts oxygen delivery to the myocardium. This can manifest as silent ischaemia, angina pectoris, myocardial infarction or even heart failure.

1.1.1 Epidemiology

CAD is a leading cause of morbidity and mortality worldwide. In the United Kingdom (UK) there has been a drive to combat this disease over the last couple of decades. Health care initiatives and policies have made cardiovascular disease a key priority (Department of Health and Great Britain, 2000). In the 1970's mortality from CAD was gradually decreasing, however a steeper decline ensued after the 1990's (Scarborough *et al*, 2010). This decline has been achieved through better education, identification and treatment of the modifiable risk factors such as hypertension, diabetes mellitus, cigarette smoking and dyslipidaemias (Unal, Critchley and Capewell, 2004). Also, the introduction of percutaneous coronary intervention (PCI) into the mainstream treatment plans for acute myocardial infarction has changed the face of the disease. The number of prescriptions for cardiovascular medications has soared, a six-fold increase from 1986 to 2011 (Townsend *et al*, 2012). Similarly, the number of interventional procedures performed has increased with most centres providing twenty four hour catheter laboratory services for primary PCI. Despite the restructuring of health care resources, CAD remains a leading cause of mortality in the UK. This situation is further compounded, by the fact that there are communities within the UK with worse mortality rates than the overall UK population (Gill *et al*, 2007). The South Asian community accounts for 4% of the UK population and represents individuals whose ancestral roots span from India, Pakistan, Bangladesh or Sri Lanka. It is this community that has 40% higher rates of mortality from CAD than their Caucasian counterparts (Gill *et al*, 2007). In addition, South Asians develop CAD at an earlier

age and tend to have a greater burden of multi-vessel disease than Caucasians (Joshi *et al*, 2007). This has spurred research into understanding and identifying potential causes for these stark differences.

The key driver behind this excess risk lies with a disproportionately high prevalence of diabetes in the South Asian community compared with the Caucasian community, with 1 in 5 South Asians being affected. The macrovascular, as well as microvascular complications of diabetes are well documented. This dysglycaemic state is closely intertwined with metabolic syndrome (Grundy, 2006), which encompasses any three of the following factors; abdominal obesity (waist circumference≥102cm in men and ≥88cm in women), raised serum triglycerides (≥1.7mmol/L), reduced high density lipoprotein (HDL) levels (HDL<1mmol/L in men and HDL<1.3mmol/L in women), raised blood pressure (≥130/85) and impaired fasting glucose (≥5.6mmol/L). In the UK, the prevalence of metabolic syndrome amongst the South Asians is considerably higher compared to their Caucasian counterparts and is likely to contribute to their excess CAD risk (Tillin *et al*, 2005). There are a number of theories behind the increasing prevalence of diabetes. Neel hypothesised that diabetes was the results of the thrifty gene hypothesis, whereby insulin would be rapidly released at times of food abundance, enabling the storage of fats, only to be mobilised and catabolised at times of food scarcity (Neel, 1962). However, this theory has been slowly discredited, since its conception back in the 1960's, as diabetes was initially deemed to be a single gene defect, but is now a complex metabolic process involving various gene-environment interactions (Grarup and Andersen, 2007).

The encapsulation of South Asians as one homogenous entity is flawed. The South Asians community is a heterogeneous collection of individuals with various customs, religions and cultural habits. This diversity will be naturally reflected in their dietary habits. Generally, South Asians tend to have diets perceived to be deficient in fibre, but surplus in carbohydrates, with this imbalance in carbohydrates contributing to a greater extent towards a hyperinsulinaemic state (Misra *et al*, 2009). Despite vegetarianism being more common amongst South Asians, compared with the natives in the UK, their diets are much lower in vegetables and fruit, with a predilection towards dairy products such as yogurts

which are higher in saturated fats (Misra *et al*, 2009). The use of 'ghee' a clarified form of butter and the overzealous use of cooking oils in the preparation of dishes has been purported to be atherogenic. Both of which contains cholesterol oxides which contribute to the development of atherosclerosis (Jacobson, 1987).

The knowledge base for cigarette smoking in the aetiology of CAD is exhaustive. Smoking patterns vary considerably within the South Asians community, with those of Bangladeshi origin having the highest rates (Bhopal *et al*, 1999). However, when factored into the entire South Asian cohort, the overall smoking rates tend to be much lower, comparatively to those for the Caucasians in the UK.

Hypertension is a well-known cause of CAD. There is however, some discordance as to whether hypertension rates amongst South Asians are higher compared to Caucasians. Studies by McKeigue and Cappucio revealed that hypertension was more prevalent in the South Asian community in the UK than their European counterparts (McKeigue, Shah and Marmot, 1991; Cappuccio *et al*, 1997). In contrast, Lane et al. reported that hypertension rates were comparable between South Asian men and Caucasian men (Lane, Beevers and Lip, 2002). Despite this conflict in the literature, the occurrence of hypertension in the South Asian community translates to a higher cardiovascular risk than for Caucasians with hypertension (Cappuccio *et al*, 1997).

Lipoproteins are causally linked with CAD, with the HDL conferring protection and the low density lipoprotein (LDL) conferring atherogenicity. There is growing evidence supporting ethnic differences in lipoprotein profiles. Individuals from the South Asian subcontinent tend to have lower HDL levels and higher levels of triglycerides, compared with their European counterparts, which follows the trend observed in diabetes mellitus (McKeigue, Shah and Marmot, 1991; King, Aubert and Herman, 1998). Furthermore, South Asians have more severe CAD, despite lower or comparable cholesterol levels, suggesting an increased susceptibility to atherosclerosis for given cholesterol levels (Lowry *et al*, 1983). The role of lipoproteins in the aetiology of CAD is well founded, by understanding the ethnic differences in such entities may help to further explain the excess risk observed in the South Asians community.

1.2 Lipoproteins

1.2.1 Introduction

Lipoproteins are dynamic particles that are primarily concerned with the transports of lipids to the tissues of the body. Lipoproteins are spherical and consist of an outer casing of proteins and amphipathic lipids, with an inner hydrophobic core of sequestered cholesterol and triglycerides. The proteins that are attached are known as apolipoproteins (apo), which contribute to the structural stability of the entire particle as well as assisting in the solubilisation of the inner core Figure 1.1.

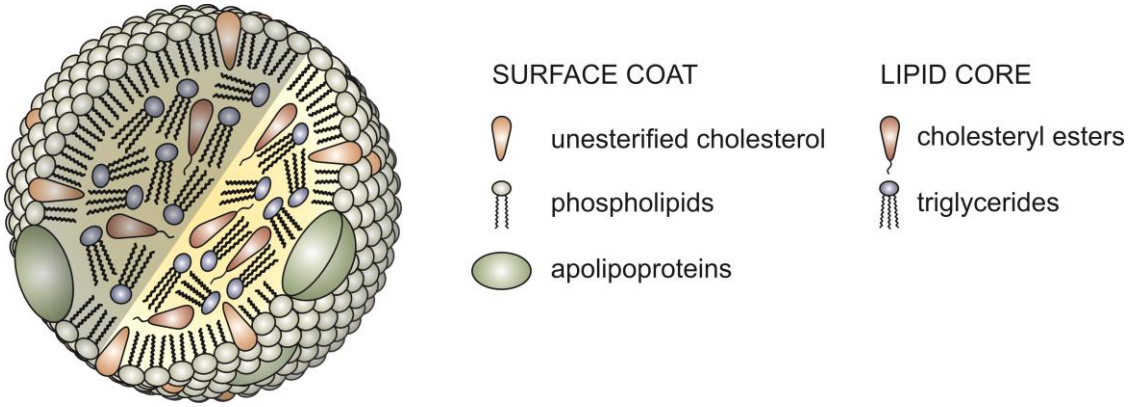


Figure 1.1 Composition of a lipoprotein particle, (AntiSense, 2010)

Lipoproteins can be simplistically categorised according to their constituent apolipoprotein into apoA and apoB containing lipoproteins. ApoA is contained within HDL particles, whereas apoB is contained within the LDL, very low density lipoprotein (VLDL) and chylomicron particles (Rader and Hobbs, 2005).

1.2.1.1 **ApoA**

ApoA-1 is the largest contributor to the total molecular mass of the HDL particle (70%), with apoA2 contributing a smaller proportion (15%) (Hedrick *et al*, 2001). The remainder is comprised of enzymes such as lecithin-cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP) and other low abundant proteins (Barter *et al*, 2004).

ApoA1 is synthesised and secreted in the liver; upon acceptance of lipid it becomes immature nascent HDL. This particle is discoidal owing to a phospholipid bilayer with antiparallel orientated apoA1 molecules (Segrest *et al*, 1999). The C-terminal of apoA1 is concerned with lipid binding and facilitates the internalisation of phospholipids and cholesterol into the nascent HDL particle, transforming the particle into mature spherical HDL (Saito, Lund-Katz and Phillips, 2004; Tall *et al*, 2008; Eisenberg, 1999).

1.2.1.2 **ApoB**

ApoB100 is the primary structural component of LDL and VLDL, with its smaller isoform, apoB-48 found within chylomicrons. The largest contribution by protein mass of LDL and VLDL is ApoB100, with a small percentage comprised from various low abundant proteins such as calgranulin A, lysozyme, ApoCIII and ApoH (Segrest *et al*, 2001; Karlsson *et al*, 2005).

The LDL particle is spherical and only contains one molecule of apoB100 (Schumaker, Phillips and Chatterton, 1994). The apoB100 consists of α helices and β pleated sheets (Schumaker, Phillips and Chatterton, 1994). A critical feature of apolipoproteins is their ability to transfer and exchange between different particles, mediating different functions. ApoB100 suffers from a lack of exchangeability, which is imparted by the β structure, which tethers the protein (Segrest *et al*, 1992).

Interestingly, lipid binding is mediated by both α helices and β sheets, but to varying extents. The β structure confers constant lipid affinity, whilst the α helices confers alternating lipid affinity, which are critical in the maturation of nascent VLDL to mature VLDL and ultimately to LDL particle (Segrest *et al*, 1992; Segrest *et al*, 1994).

LDL is cleared from the circulation through receptor mediated endocytosis via the LDL receptors in the liver. It is a proline rich domain near the C-terminal of the apoB100 that facilitates receptor binding (Yang *et al*, 1986).

1.2.2 Classification of Lipoproteins

1.2.2.1 Introduction

As alluded to previously, the lipoproteins can be classified according to their apolipoprotein scaffold, however they can be further sub classified into discrete domains by exploiting their various physicochemical attributes, such as density or electrophoretic mobility (Alaupovic, Lee and McConathy, 1972).

1.2.2.2 Classification of HDL

The classical method used for classifying the HDL particle is by density based techniques. The particle can be separated into two subspecies, HDL2 and HDL3. The HDL2 particles (density 1.063-1.125g/mL) are buoyant and large, a reflection of their low protein to lipid ratio (1:2). By contrast, the HDL3 particles (d 1.125-1.21g/mL) are denser and smaller, due to a higher protein to lipid ratio content (10:1) (Kontush and Chapman, 2006). Interestingly, apoA1 is exclusively present in HDL2 subspecies, whilst both apoA1 and apoA2 are present in HDL3 subspecies (Eisenberg, 1999).

Furthermore, the electrophoretic properties of the HDL particles can also be exploited to give rise to five distinct sub-species of reducing size; HDL2b, HDL2a, HDL3a, HDL3b, and HDL3c (Rosenson *et al*, 2011) Figure 1.2.

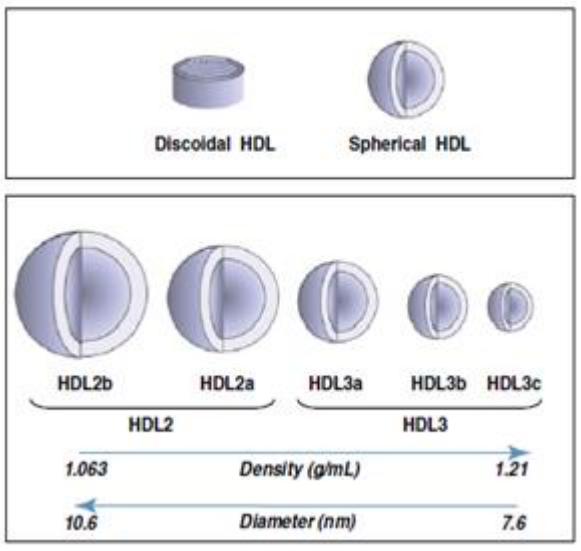


Figure 1.2 HDL particle subpopulations (Camont, Chapman and Kontush, 2011)

1.2.2.3 **Classification of LDL**

Similarly, the use of ultracentrifugation techniques revealed the LDL particle (d 1.019-1.063g/mL) to exist as a continuum of four sub fractions (LDL1 to LDL4), each with varying degrees of buoyancy and lipid content (Krauss and Burke, 1982). Sub-fraction LDL1 represents the largest and most buoyant of the subclasses, conferred by a low protein to lipid ratio. The transition from LDL1 to LDL4 is reflected by decreasing particle size, by virtue of an increasing protein to lipid ratio and a conformational change in the apoB100 structure (McNamara *et al*, 1996).

Similar to HDL, the LDL particle can be classified according to it electrophoretic mobility. Krauss revealed that the LDL particle can exist as seven distinct subclasses (Krauss and Burke, 1982).

Lipoproteins are heterogeneous entities with varying characteristics and functions. Their integral role in atherosclerosis along with disturbed expression may assist in understanding the excess burden of CAD in South Asians compared to Caucasians.

1.2.3 Lipoproteins in South Asians

1.2.3.1 **HDL in South Asians**

South Asians are known to have lower levels of HDL than Caucasians (Karthikeyan *et al*, 2009). Furthermore, South Asian subjects have smaller and denser HDL particles than Caucasian subjects. Healthy South Asian men were found to have higher levels of the smaller denser HDL3 along with lower levels of HDL2 compared to Caucasian men from the Framingham offspring study (Bhalodkar *et al*, 2004). The predominance of the smaller, denser HDL is judged to confer less cardio-protection, which may be responsible for the ethnic differences in risk (Johansson *et al*, 1991). The larger buoyant HDL is cardio-protective, as it is an acceptor of lipid and promotes the efflux of cholesterol from the periphery to the liver for breakdown, in a process called reverse cholesterol transport.

1.2.3.2 **LDL in South Asians**

Similarly, two types of LDL patterns exist, one that is predominantly composed of large buoyant LDL subspecies referred to as Pattern A and the other predominantly composed of small dense LDL subspecies, referred to as Pattern B (Berneis and Krauss, 2002). It is thought that a hypertriglyceridemic state may be the driver behind the generation of the small dense LDL subspecies (Griffin *et al*, 1994). Despite the similarity in LDL levels, the LDL particles tend to be dominated by pattern B in the South Asians compared with Caucasians (Kulkarni *et al*, 1999). Pattern B is highly atherogenic and has been shown to be adversely associated with CAD risk (Austin *et al*, 1988).

The LDL particle has enjoyed the scientific limelight for the last two decades, which has led to a greater understanding into its role and the remarkable survival benefit from its therapeutic modulation. As therapeutic measures for LDL control become exhausted, more focus will be on the HDL particle due to its athero-protective role and its potential for translatable health benefits.

1.2.4 The Roles of the Lipoproteins

1.2.4.1 **The Atherogenic role of LDL**

The concept of atherosclerosis has evolved from a simple process of lipid retention in the vessel wall to a complex inflammatory process. The behaviour of the LDL particle and its oxidised derivatives are integral to the development of atherosclerosis. The normal vascular biology is maintained by nitric oxide, which mediates vasodilatation and has inhibitory effects on platelet aggregation, leukocyte adherence and smooth muscle proliferation. This pendulum is in tight balance from the opposing vasoconstrictors, endothelin-1 and angiotensin-II. However, the inhibition of nitric oxide synthesis by LDL and the interaction of the various integrative risk factors such hypertension and diabetes, swing the pendulum towards endothelial dysfunction (Vergnani *et al*, 2000; Anderson *et al*, 1995). Endothelial dysfunction leads to a cascade of events which includes the expression of adhesion molecules on the vessel wall, facilitating the adhesion and migration of leukocytes into the subendothelial space. The sequestration of the

LDL particle into the subendothelial space renders it susceptible to oxidative modification by various enzymes, which principally include myeloperoxidase (Heinecke, 1997). The key targets for oxidative attack are the phospholipids and the amino acid residues on the apoB100 protein. Scavenger A I/II and CD36 receptors are present on the macrophages and bind to the oxidised amino acid residues and oxidised phospholipids on the LDL particles, respectively. Both scavenger receptors contribute an important pathway for the clearance of oxidised LDL (Kunjathoor *et al*, 2002). The resulting internalisation of the oxidised LDL particle by the macrophage, leads to the generation of the lipid laden foam cell, which promotes the development of a fatty plaque. Oxidised LDL along with the foam cells stimulates the release of various mediators which promote an inflammatory profile that contributes to complicated atherosclerotic plaques (Libby, 2002).

1.2.5 The Athero-protective role of HDL

The athero-protective role of HDL is based on its ability to shunt cholesterol from the periphery to the liver, which is dependent on its interaction with various receptors, but more importantly on its constituent apoA1. Lipid poor apoA1 is an acceptor of lipids and provides an essential mechanism for transfer of cellular cholesterol esters. ApoA1 has been shown to promote the efflux of cholesterol from plaques in murine knockout models but also in humans (Boisvert, Black and Curtiss, 1999; Nissen *et al*, 2003). The identification of a novel point mutation in its gene, in a small group of individuals from northern Italy, was associated with longevity (Gualandri *et al*, 1985). The therapeutic infusion of this apoA1-Milano in patients with acute coronary syndrome (ACS) resulted in significant reductions in the size of atherosclerotic plaques (Nissen *et al*, 2003).

One of the key receptors for promoting the externalisation of cholesterol to the lipid poor apoA1 is the ATP binding cassette A1 (ABCA1) receptor (Neufeld *et al*, 2001). This receptor is expressed in the intestines, liver and macrophages (Fitzgerald *et al*, 2001). The role of the ABCA1 receptor in reverse cholesterol transport was best highlighted in transgenic mice, whereby an over expression of

this receptor led to higher HDL and apoA1 levels with a concomitant reduction in atherosclerosis (Vaisman *et al*, 2001; Joyce *et al*, 2002).

The ABCG1 is another membrane spanning ATP receptor, which transfers cholesterol to the HDL particle directly, rather than to the lipid poor apoA1 in the HDL particles, as in the case for the ABCA1 receptor (Wang *et al*, 2004). The expression of both receptors is under the influence of transcription factors such as sterol response element binding factor protein (SREBP) (Horton, Goldstein and Brown, 2002; Costet *et al*, 2000). Sterols present in the macrophage stimulate transcription factor expression and ultimately the expression of both ABCA1 and ABCG1 receptors (Janowski *et al*, 1996). Recent interest has focussed on the non-protein coding segments of RNA called microRNA (miRNA), which modulate gene expression (Rayner *et al*, 2010). An example is miRNA-33, which is located on intron 16 of the SREBP gene and has been shown to reduce ABCA1/ABCG1 receptor expression, consequently reducing cholesterol efflux. The development of an antagonist to this miRNA-33 increased expression of both ABCA1 and ABCG1 receptors with a consequential rise in HDL levels (Rayner *et al*, 2010).

Furthermore, the scavenger receptor B1 (SRB1) is a scavenger receptor that is expressed in steroidogenic tissue and is concerned with shuttling of cholesterol esters to the HDL particle for catabolism (Acton *et al*, 1996). Ji showed that high expression of this SRB1 receptor resulted in a threefold increase in cholesterol clearance (Ji *et al*, 1997).

1.2.6 The lipoproteins as targets for therapy

1.2.6.1 LDL decreasing drugs

Dyslipidaemia is a causative factor in the development and progression of CAD. The first landmark trial tackling this risk factor was the Scandinavian Simvastatin Survival Study (4S), which provided robust clinical evidence for the use of simvastatin in the care of CAD patients, achieving impressive reductions in total cholesterol and LDL of 25% and 35% respectively ('Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the

Scandinavian Simvastatin Survival Study (4S)', 1994). It was also shown to have some effect on HDL, by increasing its levels by 8%. The significance of this trial was attributable to the huge survival benefit in the CAD patients over the course of the trial. This revolutionised the way in which CAD patients were managed, becoming the mainstay of primary and secondary prevention regimens.

In another landmark trial called Pravastatin or Atorvastatin Evaluation and Infection Therapy Thrombolysis in Myocardial Infarction 22 (PROVE-IT TIMI 22), the efficacy of high dose 80mg atorvastatin was compared with standard dose of 40mg pravastatin in patients post ACS (Cannon *et al*, 2004). The high dose of atorvastatin achieved much lower median LDL levels compared with pravastatin (1.60 mmol/L vs. 2.46 mmol/L respectively) from a baseline of 2.74 mmol/L. Atorvastatin was shown to significantly reduce the primary end point of death and major cardiac events, by 16 % compared with the pravastatin group. This evidence led to adoption of higher doses of statins, in particular atorvastatin, to achieve lower LDL targets in the setting of ACS (Cannon *et al*, 2004).

The evidence for lower LDL levels is well founded such that a 1 mmol/L reduction has been shown to correspond with a 21% reduction in CAD risk (Cholesterol Treatment Trialists' (CTT) Collaborators *et al*, 2012).

Furthermore, statins in particular atorvastatin has been shown to reduce the levels of the small dense LDL subspecies, which are known to confer increased CAD risk via the up regulation of the LDL receptor and a reduction in precursor availability (Guerin *et al*, 2000).

Another class of hypolipidemic drugs are the fibrates which activate peroxisome proliferator-activated receptor (PPARα). Fibrates reduce triglycerides and their enriched lipoproteins, which also reduces the formation of small dense LDL particles (Ikewaki *et al*, 2004). Despite their favourable changes to the lipoprotein profile, their clinical use has been limited due to controversial side effects and a lack of clinical efficacy in various randomized clinical trials(Saha *et al*, 2007).

1.2.7 HDL increasing drugs

Recently, there has been tremendous focus on using drugs to increase HDL levels, in support of previous epidemiological evidence (Gordon *et al*, 1977). This niche in the market is potentially lucrative, especially in the secondary prevention of myocardial infarction, as statins lower LDL with some effect on HDL (Jones *et al*, 2003). Drugs such as niacin and newer agent such as CETP inhibitors have been shown to specifically improve HDL levels. Niacin improves HDL levels by reducing the catabolism of apoA1, but also induces favourable changes on the apoB containing lipoproteins, primarily by reducing the synthesis of triglycerides. The reduced availability of triglycerides reduces the potential for atherogenic transformation of the LDL particles into small dense subspecies (Kamanna and Kashyap, 2000; Jin, Kamanna and Kashyap, 1999). In hypercholesterolemia patients, niacin (2g) increased HDL by 23% and reduced both LDL and triglycerides by 14% and 29% respectively (Morgan *et al*, 1996). A doubling of the dose was shown to mediate a protective shift in the HDL particles, by preferentially increasing the levels of large buoyant HDL2 by 135%, without any effect on HDL3 levels (Wahlberg *et al*, 1990). However, niacin failed to demonstrate any clinical efficacy in the AIM-HIGH trial, when it was added to high intensity statin therapy in the patients with established cardiovascular disease, despite favourable changes in the lipid profiles (AIM-HIGH Investigators *et al*, 2011).

The most recent instalment in the HDL boosting story is the CETP inhibitors. CETP is an enzyme that is involved in the shunting of cholesterol esters between the HDL particles and the apoB containing lipoproteins. The first generation CETP inhibitor was torceptrapib, which showed impressive HDL boosting properties in its phase 1 trial (Clark *et al*, 2004). However, in the ILLUMINATE trial (phase 3) torceptrapib had an adverse off-target effect on the mineralocorticoid axis, increasing systolic blood pressure and adversely increasing mortality, despite improvements in HDL levels (Barter *et al*, 2007b). Next generation CETP inhibitors were developed which included; dalceptrapib and anaceptrapib. In animal studies, dalceptrapib induced changes in the HDL levels, preferentially targeting HDL2 (Okamoto *et al*, 2000). In the dal-VESSEL study (phase 2b), dalceptrapib increased

HDL levels, without the controversial increase in blood pressure (Luscher *et al*, 2012). Despite the improved side effect profile, dalceptrapib failed to improve clinical outcomes in patients with ACS in the dal-OUTCOMES study (phase 3) (Schwartz *et al*, 2012). The fate of the CETP inhibitors is yet to be decided, as the results from the REVEAL study, which explores the clinical utility of anacetrapib, in patients with established vascular disease, are eagerly anticipated.

The failure of the niacin and the latest CETP inhibitors has reopened the longstanding debate that the quality of the HDL particle may be more important than its quantity. The fixation with an arbitrary level has neglected the functional aspects of the particle. The proteins attached to HDL include common proteins such as the apoA1, but also low abundant proteins which have properties concerned with redox, inflammation and complement regulation. Such low abundant proteins may confer a cardioprotective role that is mediated by the HDL particle. The differential expression of the low abundant proteins may help to explain the excess CAD risk observed in the South Asians compared with the Caucasians.

1.2.8 Mediators of the atherogenic role of LDL

The exploration of the distinct proteins contained within the different lipoprotein classes will further our understanding of how they behave in health and disease and lead to new disease markers. The protein content of LDL accounts for 25% of the overall mass, with the majority attributable to apoB100. However, a number of low abundant proteins have been identified that mediate specific atherogenic functions and it is these that may be differentially expressed, causing the differences in CAD risk between the two ethnicities, despite the comparable LDL levels.

A novel protein called calgranulin A, also known as S100A8 has been identified on the LDL particle (Karlsson *et al*, 2005). This protein belongs to the S100 family which is concerned with calcium homeostasis, cell differentiation and cytoskeletal reorganisation (Vogl *et al*, 2004). It is expressed both in the cytoplasm and nucleus of a number of cells, in particular neutrophils and monocytes (Nacken *et al*, 2003).

It is released by activated leukocytes and cytokines released from vascular injury (Rammes *et al*, 1997). Calgranulin A activates a receptor for advanced glycation end products (RAGE), which mediates downstream inflammatory and thrombotic effects (Hofmann *et al*, 1999). The significance of this protein was highlighted in the PROVE IT TIMI 22 trial, whereby calgranulin A levels were significantly higher in ACS patients who died or re-infarcted at 30days compared to event free survivors (Morrow *et al*, 2008). The use of this protein as a possible prognostic biomarker was advocated by strong evidence, showing that it was predictive of further cardiovascular events after the initial ACS event. The utility of this marker provided superior prognostic information even after adjusting for C-reactive protein (CRP), such that the individuals in the highest quartile of calgranulin A had a two-fold increased risk of cardiovascular death or re-infarction (Morrow *et al*, 2008). The simplistic passive notion that the LDL particle becomes trapped in the sub-endothelial space and becomes oxidised is evolving, to encompass a more active role in inflammation.

Although, apoB100 contributes significantly to the overall protein content of LDL, other apolipoproteins also exist due to their exchangeability. ApoC3 a small glycoprotein that is synthesised in the liver, is contained on the LDL particle, but mainly on the VLDL particle (Karlsson *et al*, 2005). It has gained interest of late, due to its inhibitory effects on both lipoprotein lipase and receptor mediated clearance of the triglyceride rich lipoproteins, thereby promoting hypertriglyceridaemia. A number of single nucleotide polymorphisms (SNP) in the promoter region of the apoC3 gene have been identified, which alter its expression. Under normal conditions, apoC3 gene expression is suppressed by insulin, however polymorphisms such as 455T>C, overcome the inhibitory effects and leads to elevated levels of apoC3 in the presence of exaggerated insulin levels (Hegele *et al*, 1997; Chen *et al*, 1994). The close interplay between insulin resistance and hypertriglyceridaemia in metabolic syndrome suggests that apoC3 may be implicated. The combination of the 455C polymorphism leading to elevated levels of apoC3, in the presence of metabolic syndrome has a synergistic effect in conferring increased CAD risk (Olivieri *et al*, 2003). The prognostic capability of apoC3 was examined in the cholesterol and recurrent events (CARE)

trial where it was associated with a two-fold increase in CAD risk in acute MI patients (Sacks *et al*, 2000). The incidence of metabolic syndrome in the South Asian community is considerably higher compared with their Caucasians counterparts. It is therefore plausible that apoC3 levels may be higher in the South Asians, reflecting greater atherogenicity and greater CAD risk.

ApoH is a glycoprotein of hepatic origin and circulates in the bloodstream in an unbound form, but can associate with negatively charged molecules such as phospholipids. ApoH has strong affinity for the oxidised LDL rather than native LDL and forms a highly immunogenic complex (Hasunuma *et al*, 1997). Surprisingly, apoH has both protective and harmful effects on atherosclerosis. The anti-atherosclerotic properties arise from its ability to inhibit the uptake of oxidised LDL by the macrophage (Hasunuma *et al*, 1997). In contrast, its co-incubation with the anti-phospholipid antibody has the reverse effect, with enhanced macrophage endocytosis mediating a pro-atherogenic tendency (Hasunuma *et al*, 1997). Immunisation with anti-ApoH antibodies in LDL receptor deficient mice resulted in greater atherosclerotic lesions than control mice (George *et al*, 2000). Clinically, anti-apoH antibodies were predictive of stroke and myocardial infarction in subjects followed up over 20years (Brey *et al*, 2001). This pro-thrombotic characteristic of the auto-antibodies is similar to that observed in Antiphospholipid Syndrome. The role of apoH may be protective; however, the immune response to the oxidised LDL/ApoH complex may be deleterious and may confer cardiovascular risk. Greco showed that anti-apoH was associated with increasing CAD severity (Greco *et al*, 2010). Therefore, it is possible that the levels of the apoH/oxidised LDL complex and its associated autoantibody, may be higher in the South Asians compared with Caucasians, reflecting the greater burden of CAD.

Lysozyme is a hydrolytic enzyme concerned with host defence and has been identified on the LDL particle (Sava, 1996). A study by Welman in the 1980's sought to determine the levels of this hydrolytic enzyme during the acute presentation of MI in presumably Caucasian subjects (Welman *et al*, 1980). They reported elevated levels of lysozyme, which peaked at day 3, reflecting an inflammatory response to the insult (Welman *et al*, 1980). Similar findings were

observed in a cohort of South Asian subjects with ACS (Chavan, Patil and Karnik, 2007). In diabetic subjects the LDL particles were found to be enriched with lysozyme compared with LDL particles from control subjects (Pettersson *et al*, 2011). The chronic hyperglycaemia in diabetes leads to glycosylated tags on various amino acids and lipid residues, which are called advanced glycation end products (AGEs). Lysozyme binds with high affinity to these AGEs, consequently reducing their clearance and also rendering itself dysfunctional to engage in host defence (Li, Tan and Vlassara, 1995). The reason for the lysozyme enrichment was probably due to greater AGE modification on the LDL particle of diabetics than controls (Pettersson *et al*, 2011). The increasing prevalence of metabolic syndrome and diabetes amongst the South Asian community may lead to greater AGE modifications in the LDL particle and reflect greater lysozyme content compared with Caucasians.

Lipoprotein (a) represents an apoB100 molecule bound to apo(a) by a disulphide bond. Its density falls within the range for LDL and is therefore isolated in the LDL fraction when using density based methods. Lipoprotein (a) has gained considerable interest over the last decade, due to its strong association with CAD, with patients in the upper quartile of lipoprotein (a) exhibiting a three-fold increased risk of CAD, compared to patients in the lower quartile (Imhof *et al*, 2003). This adverse risk is likely to be attributable to its sequence homology with plasminogen, resulting in competitive inhibition of receptor binding and impairing fibrinolysis (Aznar *et al*, 1993). Interference in this pathway is augmented by the release of plasminogen activator inhibitor-1 (PAI-1), which contributes to a pro-thrombotic state. Compared to Caucasians, South Asians were found to have higher levels of lipoprotein (a) and PAI-1 levels despite lower rates of subclinical atherosclerosis (Anand *et al*, 2000a). Therefore, the higher levels of this pro-thrombotic mediator would translate to a higher CAD risk and possibly help to explain the ethnic differences.

1.2.8.1 **Mediators of the anti-atherogenic role of HDL**

More low abundant proteins have been identified on the HDL particle than the LDL particle because of its higher protein content. Of the low abundant proteins, paraoxonase-1 (PON-1) is a recognised anti-inflammatory mediator carried on HDL, which is synthesised in the liver (Durrington, 2007). PON-1's mechanism of action is the inhibition of LDL oxidation, by preventing the accumulation of lipid peroxides in the LDL particle (Mackness, Durrington and Mackness, 2000). In rodent studies, PON-1 knockout mice were highly sensitive to the effects of organophosphate toxins when compared to wild type littermates. The susceptibility to organophosphate toxicity was associated with an increase in atherosclerotic lesions in the PON-1 knockout mice (Shih *et al*, 1998). In a reverse of that experiment, PON-1 over expression led to a significant reduction in atherosclerotic lesions compared with wild type littermates (Tward *et al*, 2002). PON-1 also modulates the expression of the adhesion molecules on the vessel wall, thereby limiting leukocyte adhesion (Tward *et al*, 2002). It was revealed that patients with myocardial infarction, presumably Caucasian, had depleted levels of PON-1 compared with control subjects, hence reduced cardiac protection (Tward *et al*, 2002; Ayub *et al*, 1999). This vulnerability to such a disease may be a cause or an effect of low levels of PON-1. Sarkar et al. reported that PON-1 activity in South Asians with confirmed CAD was significantly lower compared to healthy control subjects (Sarkar, T and Madhusudhan, 2006). These findings provide sufficient evidence to support lower levels of PON-1 in CAD. In the literature, no studies to date have made comparisons between the two ethnicities in the setting of CAD, to ascertain if there is differential expression of PON-1. The higher prevalence of CAD in the South Asian community may be a reflection of greater depletion of PON-1 stores in HDL, compared to their Caucasian counterparts, leading to a greater susceptibility to the oxidative stress that occurs with acute MI.

Clusterin is a protein with antioxidant properties that has been identified in the HDL particle. Navab et al. demonstrated that a truncated protein sequence (D112-D22) of clusterin exerted similar properties to its native peptide, resulting in a 70% reduction in atherosclerotic plaques in mice (Navab *et al*, 2005). In cynomolgus monkeys, the administration of this truncated peptide caused a fall in

lipid hydroperoxides, along with a concomitant rise in paraoxonase levels, suggesting that clusterin levels may boost levels of other antioxidants such as paraoxonase (Navab *et al*, 2005). The striking feature of clusterin is its ability to minimise the cytotoxic effects induced oxidised LDL (Schwarz *et al*, 2008). A study by Hoofnagle showed that obesity had a suppressive effect on clusterin levels. It is therefore conceivable that the high prevalence of diabetes and metabolic syndrome within the South Asians may influence clusterin levels (Hoofnagle *et al*, 2010).

Serum amyloid A contributes to the distinctive protein cargo on the HDL particle. It is of hepatic origin and consists of 4 isoforms with serum amyloid-A4 accounting for 90% of the total (de Beer *et al*, 1995). Its levels can drastically increase in acute inflammation, which is orchestrated by various cytokines and in turn activates further downstream signalling mediating an amplified response (Furlaneto and Campa, 2000; Uhlar and Whitehead, 1999). It is therefore not unsurprising, that patients with ACS have higher levels of serum amyloid A. (Alwaili *et al*, 2012). The role of serum amyloid A is not simply confined to inflammation, but has overlap with the clotting cascade, as it was shown to promote the release of tissue factor in human endothelial cells, causing a hypercoagulable state in ACS patients (Song *et al*, 2009; Zhao, Zhou and Heng, 2007). There is some suggestion that serum amyloid A may be partially controlled by feedback mechanisms within HDL, which limit the basal secretion of lipopolysaccharide cytokines such as TNF alpha, IL-1 beta and IL-8 (Furlaneto and Campa, 2000). The lipoprotein profiles of patients with CAD are dominated by low HDL, hence low levels would exert less feedback control over serum amyloid A, leading to enhanced inflammation (Franco, Sandri and Campa, 2011). The lower levels of HDL in South Asians compared to their Caucasian counterparts, may cause differential serum amyloid A expression, which may contribute to their background of subclinical inflammation (Anand *et al*, 2000b).

Platelet activating factor (PAF) is an active phospholipid, which is present in the inflammatory cells involved in the anaphylactic reaction (Hanahan, 1986). Endothelial derived PAF plays an integral role in the adhesion of leukocytes to the vessel wall, thereby contributing to the inflammatory process (Zimmerman *et al*,

1990; Zimmerman, McIntyre and Prescott, 1985). Interestingly, HDL has been shown to contain the antidote to this inflammatory mediator, by way of platelet activating factor acetylhydrolase (PAH-AH), which removes the acetyl group from PAH rendering it inactive (Blank *et al*, 1981). Sugatani demonstrated that HDL inhibited PAF expression in a dose dependent manner, reinforcing its role as an anti-inflammatory mediator owing in part to its carriage of PAF-AH (Sugatani *et al*, 1996).

The HDL particle possesses a number of pro-inflammatory and anti-inflammatory mediators. Therefore, the predominance of certain mediators will determine the fate of the particle in terms of its anti-atherogenic or pro-atherogenic potential. Subjects of South Asian origin tend to have higher levels of subclinical inflammation measured using markers such as CRP compared with Caucasians (Chambers *et al*, 2001). Therefore, in the setting of CAD the HDL particle is likely to become dysfunctional with the predominance of pro-inflammatory mediators. The South Asian subjects may have an up-regulation of pro-inflammatory mediators contained within the HDL compared with the Caucasian subjects.

1.2.9 Lipoprotein separation

Lipoproteins are a heterogeneous group, with diverse physicochemical properties. Historically the cornerstone for isolating lipoproteins was based on their density. The initial work by Havel paved the way for effectively isolating the lipoprotein using sequential ultracentrifugation (Havel, Eder and Bragdon, 1955). In this technique, the density of the serum was increased by the addition of potassium bromide solution (d 1.346 g/mL) to give a final density of 1.063 g/mL. The sample was then subjected to ultracentrifugation whereby the diffusive and centrifugal forces caused the particles to separate according to their density. Lipoproteins with densities less than that of the solvent would sediment in the top of the tube, enabling easy extraction. This process of lipoprotein floatation was then repeated with varying densities of salt solution until the desired lipoprotein fraction was collected (Havel, Eder and Bragdon, 1955). The basis of this work led to the creation of density centric definitions for the different lipoprotein classes.

The main set back to this process of separation is the long centrifugal times. Various researchers have designed ways in which to streamline this process. Instead of isolating the lipoproteins with sequential increasing densities of potassium bromide, Chapman et al. exposed the plasma to a discontinuous gradient of potassium bromide in a single centrifugal run (Chapman *et al*, 1981). This achieved impressive separation of the lipoprotein classes in a fraction of the time (Chapman *et al*, 1981).

There was growing consensus that the use of potassium bromide for lipoprotein isolation would potentially hinder protein determination. The ionic strength of the salt buffer was postulated to interfere with the weak hydrophobic bonds between the exchangeable apolipoproteins, therefore leading to protein desorption and an underestimation of the actual protein content in the classes.

This spurred the development of novel methods, which avoided exposing the lipoproteins to such salts. Graham et al. described a method for separating plasma lipoproteins using a self-generating gradient of iodixanol coupled with ultracentrifugation (Graham *et al*, 1996). Iodixanol was chosen for its non-ionic properties. Essentially, serum was mixed with iodixanol and then overlaid with buffered saline and centrifuged for 3hrs at 16°C. This method compared favourably with Havel's sequential density ultracentrifugation, with good separation of the lipoprotein classes (Havel, Eder and Bragdon, 1955). Interestingly, the density definitions for the lipoprotein classes were slightly narrower than those defined by sequential density ultracentrifugation, due to the hyperosmolar nature of potassium bromide drawing water into the fractions and expanding the density range (Graham *et al*, 1996).

More recently a novel method was developed by Stahlman et al. using deuterium oxide/sucrose solutions for isolating the lipoproteins fractions for proteomic discovery (Stahlman *et al*, 2008). The physiological neutrality of the working solutions was a significant advantage. In principal, the methodology was very similar to that used for sequential density ultracentrifugation with a series of increasing densities of deuterium oxide/sucrose but with higher centrifugal forces, a higher ambient temperature of 30°C and with runs time between 3hours and

15hours. The lipid content for the different lipoprotein fractions were comparable between this technique and those determined by sequential density ultracentrifugation. However, the protein contents for both LDL and HDL were higher in the deuterium oxide/sucrose technique, compared with the sequential density ultracentrifugation technique that used potassium bromide. This was corroborated by mass spectrometric analysis, which revealed richer mass spectra for the LDL and HDL fractions, suggesting a more accurate assessment of the true protein content compared with the conventional sequential density ultracentrifugation (Stahlman *et al*, 2008). The barrier to its incorporation into mainstream practice is the high cost associated with deuterium oxide, however as the authors alluded to, this would be offset against richer mass spectra and a better assessment of the true protein cargo on the HDL particle (Stahlman *et al*, 2008).

Despite the different media, the above methods are still heavily dependent on ultracentrifugation. This process generates high shear forces that can potentially strip the exchangeable proteins from the lipoprotein surface. Research has thus focussed on developing newer methods for isolating the lipoproteins based on alternative physicochemical properties.

A novel agent that is used commercially by the biopharmaceutical industry as a cleanup process, has revealed potentially interesting lipophilic properties. Lipid removal agent (LRA) is a synthetic agent, which is produced by a hydrothermal reaction between hydrated lime and diatomite, creating a crystalline structure very similar to naturally occurring calcium silicate hydrate. The commercial utility of this resin lies in the removal of endotoxin, which represents lipopolysacharide molecules attached to the cell wall of gram negative bacteria. Gram negative bacteria are essential in the generation of recombinant DNA, however contamination of endotoxin poses a real challenge due to its thermostability (Sharma, 1986). The failure of adequate removal of endotoxin can potentially cause septic shock for the recipient. Purification steps using anion exchange has been shown to achieve good removal of endotoxin, but remains expensive (Hirayama and Sakata, 2002). LRA represents a plausible and cost-effective purification adsorbent compared to the other techniques (Winters *et al*, 2003).

Zhang et al showed that LRA removed up to 60% of endotoxin from an aqueous solution and was enhanced to 99.9% removal by the incorporation of NaCl (Zhang *et al*, 2005).

The lipophilic properties of this agent were explored in studies by Gordon (Gordon *et al*, 2010). Initially, HDL was isolated according to its particle size using gel filtration chromatography and subsequently co-incubated with LRA for protein enrichment (Gordon *et al*, 2010). In brief, gel filtration chromatography relies on a series of gel filtration columns that are packed tightly with beads which facilitates the separation of proteins according to their size. The small proteins are able to penetrate deep into the columns leading to longer elution times, whilst the larger proteins are unable to permeate through the pores leading to shorter elution times. This type of separation is ideal as it is relatively fast and achieves good separation with minimal sample losses. The combination of such techniques, resulted in the identification of 14 novel proteins expanding the coverage of the HDL proteome compared with sequential density ultracentrifugation (Gordon *et al*, 2010). This raised the scientific bar and suggested that the HDL proteome may be larger than initial reports, with greater need for further exploration and characterization.

Gordon followed on from his initial work using gel filtration chromatography and compared it with anion exchange chromatography and isoelectric focussing (Gordon *et al*, 2013). Each of these isolative techniques exploited a specific physicochemical property of the lipoproteins. In anion exchange, the high pressure liquid chromatography (HPLC) columns were filled with an anion resin such as DEAE-Sephadex, which would bind to the negatively charged proteins. Elution of the proteins occurred using a gradient of sodium perchlorate, which was dependent on the size of the charge i.e. the larger the negative charge the longer the retention time. Isoelectric focusing separates proteins according to their isoelectric point, the point at which the overall charge is zero. The resulting isolated fractions from the three techniques were then subjected to the LRA for sample enrichment, with subsequent digestion on the resin and final analysis on the mass spectrometer (MS). The entire spectrum of lipoproteins from VLDL to

HDL was analysed. Unsurprisingly, the chromatograms were different between the different techniques, a result of exploitation of different chemical attributes of the lipoproteins. Anion exchange chromatography yielded the highest protein hits, with the lowest protein hits attained using isoelectric focusing. Despite the discrepancies, 76 proteins were common to all three techniques. The vast majority of the proteins identified were within the density range of HDL which is consistent with its largest protein content of all the lipoproteins (Gordon *et al*, 2013). Gordon reliably isolated the HDL fractions as well as other fractions, giving greater insight into their dynamic and collaborative roles. Gordon suggested that lipoproteins may operate in unison forming subspecies mediating specific function rather than in isolation, in their distinct density based fractions (Gordon *et al*, 2013). This concept is particularly interesting, as more information can be acquired by examining the entire lipoproteins spectrum, rather than by simply analysing the HDL proteome. By overlooking the smaller proteomes conferred by the other species, such as LDL and VLDL, potentially novel proteins may be overlooked from discovery work, that may provide greater insight into disease processes.

1.2.10 Lipoproteomics

The qualitative and quantitative information pertaining to the low abundant proteins and the oxidised residues on the LDL and HDL particles can be analysed using proteomics. The term proteomics was first conceptualised by Wilkins, who sought to determine the function, regulation and expression of proteins in a biological system (Wilkins, 2009). Proteomics is an area of research that is undergoing rapid evolution, similar to the trajectory that genomics underwent in its early stages. This has been partly achieved by better sample preparation techniques, better separative capacities and better resolution of the MSs. However, the major challenge facing proteomics is the type of tissue material selected for analysis. The tissue material is very important, as its complexity in most cases can overwhelm the detection capabilities for most MS, thereby limiting protein discovery. For example, the plasma proteome is the most complex tissue material, as it contains greater than 10^6 proteins with concentrations greater than 12 orders

of magnitude. The presence of high abundance proteins such as albumin, hinder any meaningful interpretation, as they mask the signal generated from the low abundance proteins. The resolving power of most MS can cover up to 4-5 orders of magnitude (Wang and Hanash, 2005). Therefore, a number of pre-fractionation techniques need to be employed, to bring the dynamic range of the sample down, so that the MS can identify the low abundant proteins. Of the different lipoproteins, HDL is by far the most interesting, as it contains the largest protein mass. The HDL proteome has so far been shown to contain approximately 80 proteins and therefore represents an ideal medium, as it avoids the complexities associated with plasma (Gordon *et al*, 2013; Vaisar *et al*, 2007).

With better pre-fractionation techniques and newer mass spectrometric capabilities, protein coverage can be increased to identify new proteins, which may confer cardiac risk.

There are two principles for proteomic analysis, bottom-up and top-down approaches. In a bottom-up proteomic approach, proteins are digested into peptides and then separated by chromatography prior to MS analysis (Chait, 2006).

In contrast, a top-down approach analyses the intact proteins directly on the mass spectrometer, without the need for digestion (Sze *et al*, 2002; Bogdanov and Smith, 2005). Both approaches have their own inherent design flaws. For example, in bottom-up proteomics, many prototypic peptides are generated for a single protein, which can further complicate the analysis of complex mixtures. Most proteins that are present in cells and the bloodstream undergo post-translation modifications as a way of mediating different functions. The digestion of these proteins in a bottom-up approach prevents the deciphering of many of these post translational modifications. In contrast, a top-down approach requires the samples to be highly purified before analysis on the mass spectrometer. In addition, there is the issue of cost, the MS capable of analysing intact proteins such as fourier transform ion cyclotron resonance (FTICR) are extremely expensive compared to those used for bottom-up proteomics (Zhang *et al*, 2013b).

1.2.10.1 **Principles of Mass Spectrometry**

The basis of this work utilised a bottom-up approach with the samples analysed either on a Thermo Q exactive or a Waters G2S MS. The MS is an instrument that measures the mass/charge (m/z) ratio of ionised molecules. Essentially, it consists of an ionisation source, a mass analyser and a detector.

The ionisation source serves to transform the sample into ions. The sample can be ionised by a number of methods. One such method is matrix assisted laser desorption ionisation (MALDI), which is a soft ionisation technique, created by Professor Hillenkamp in the late 1980's (Karas and Hillenkamp, 1988). The sample is initially mixed with a matrix containing an organic compound and subsequently irradiated with a UV laser. The matrix absorbs the pulsed UV rays, which then transfers the energy to the analyte causing ionisation and enabling subsequent MS analysis. The attraction towards this type of ionisation technique is that it can analyse multiple samples quickly. In addition, the data processing is relatively straightforward due to the generation of singly charged ions. The main drawback however, is that it suffers from a lack of reproducibility due to variations in sample matrix composition, pH and crystallisation times (Cohen and Chait, 1996). An alternative soft ionisation technique is electrospray ionisation (ESI) which was developed by Fenn in the 1980's (Wong, Meng and Fenn, 1988). In this technique, the analyte is dissolved in a solvent and is passed through a fine capillary. The tip of the capillary is exposed to an electric current which serves to nebulise the liquid into a mist of droplets. The molecules in solution pass to ions in the gas phase under vacuum and are transmitted using a series of electromagnetic fields, which facilitates their analysis by the MS. The multiple ions generated for each biomolecule give rise to many ion peaks which accounts for the high sensitivity of ESI, which can often hinder data analysis due to the rich mass spectra.

The ions generated from such techniques travel in a vacuum to the mass analyser. In the mass analyser the ions are separated according to their m/z. Orbitrap, Triple quadrupole (TQ) and time of flight (TOF) are examples of commonly used mass analysers that can be that can be used individually or combined to increase mass accuracy and resolving power of the MS; in such cases this is called tandem mass spectrometry (MS/MS). The orbitrap mass analyser consists of inner central

spindle electrode and an outer barrel shaped electrode. Packets of ions are injected and oscillate within the central spindle due to an increasing voltage. Eventually the orbiting ions will reach their harmonic trajectory, which is relative to their m/z (Makarov, 2000). In contrast, the quadrupole consists of 4 parallel cylinders which exert an oscillating current affecting the trajectory of the ions. TQ consists of a set of three consecutive quadruple formations, the first and last quadrupole formations filter the ions according to a certain m/z whilst the central quadrupole allows collision induced dissociation of the precursor ion to take place in a bath of inert gas (e.g. argon). In TOF the ions are accelerated by an electric field towards a detector and the time taken for the ion to reach the detector determines their mass. The final section of the MS is the detector which detects the charge produced when the ion hits the surface producing the mass spectra.

Furthermore, the resolving power of the MS can be enhanced by the addition of ion mobility spectrometry (IMS). IMS is an analytical technique whereby ions are separated based on their mobility in a buffer gas, in the presence of an electric field. In travelling wave ion mobility spectrometry (TWIMS), ions initially created by ESI, are focussed and enter a drift tube and are consequently dispersed by an electrostatic field. As the ions travel down this drift tube they collide with a neutral gas, which impedes their migration and subsequent drift time. Larger ions have longer drift times than smaller ions because of a larger cross-sectional area. From the time it takes an ion to travel a known length, the drift time or an ion's mobility can be calculated (Shvartsburg and Smith, 2008). Ion mobility has been shown to increase proteome coverage by approximately 50-60% with no additional analytical time.

1.2.10.1.1 Thermo Q Exactive

The Thermo Q exactive represents the latest bench top MS, capable of very high mass accuracy and high resolution. It comprises of a quadrupole for mass filtering combined with an orbitrap as the mass analyser Figure 1.3. In brief, the ions are generated by a nano-electrospray ion source and enter the S lens, which is composed of stacked ion rings. The purpose the S lens is to increase the

transmission of the ions to the MS whilst filtering out droplets and thereby improving sensitivity. The ions travel to the quadrupole, which is capable of transferring ions of a predefined mass range to the C-trap. The ions accumulate in the C-trap and are then passed onto the higher-energy collisional dissociation (HCD) cell for fragmentation. The fragmented ions are then transferred back to the C-trap where they then travel to the orbitrap mass analyzer. The concomitant fragmentation, accumulation and detection of ions, contribute to rapid scans and improved acquisition times (Michalski *et al*, 2011).

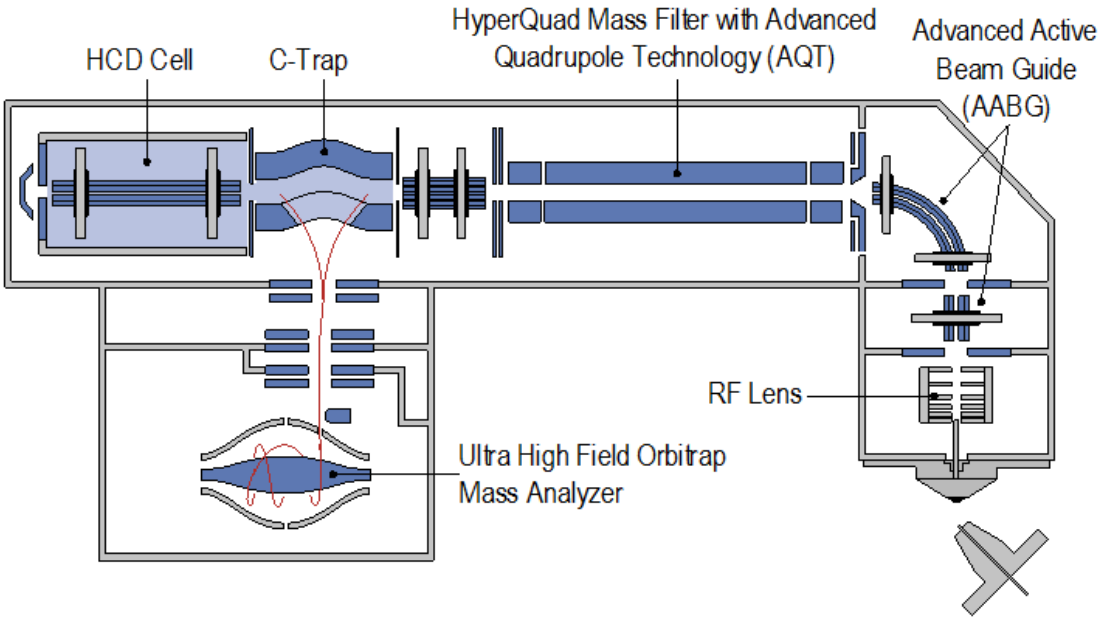


Figure 1.3 Thermo Q Exactive (*Schematic of Q Exactive MS.* 2016)

1.2.10.1.2 Waters G2S

The Waters Synapt G2S is part of the latest generation of Waters high resolution Q-ToF MSs Figure 1.4 . It features high resolution quadrupole and TOF coupled with ion mobility separation for an additional layer of separation. In brief, the ions created by ESI are transferred to the step wave ion guide, which removes the neutral ions thereby increasing the sensitivity and ion intensity. The ions are then transferred to the quadrupole, which acts as a mass filter allowing ions of a certain mass range to enter the triwave region. The triwave region is where ion mobility can be enabled. When mobility is enabled, the ions enter from the quadrupole and are gated in the Trap cell. Ions are released in packets and undergo mobility

separation according to their size, shape and charge. The ions are subsequently transferred (whilst maintaining their mobility separation) to QuanTOF for high resolution mass analysis.

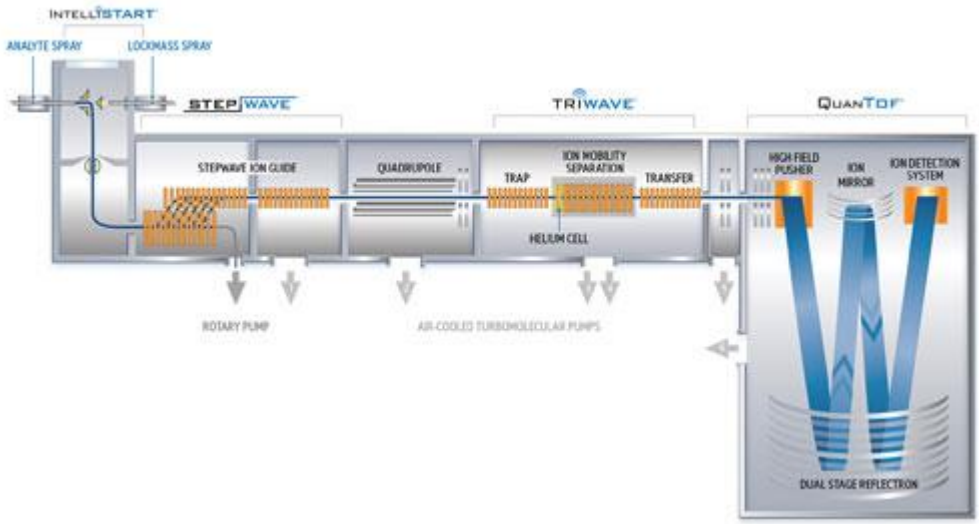


Figure 1.4 Schematic representation of the TW-IM-MS/MS-QTOF mass spectrometer.

1.2.10.2 **Data acquisition**

The data can be acquired in a data dependent or data independent fashion. In data dependent acquisition, the instrument performs a survey scan on all the peptide ions and selects the ten most abundant peptide ions for fragmentation. The main criticism to this type of acquisition is that the low abundant peptide ions are often overlooked for selection and omitted from MS/MS analysis. The bias towards the high abundant proteins for fragmentation prompted the development of the unbiased data independent acquisition.

In data independent acquisition, essentially all peptide ions within a specific range of m/z undergo fragmentation, generating MS/MS spectra and leading to the identification of more peptides (Liebler, 2004), Figure 1.5. This type of acquisition is ideal when considering that biomarker discovery is often limited to the medium and low abundant proteins.

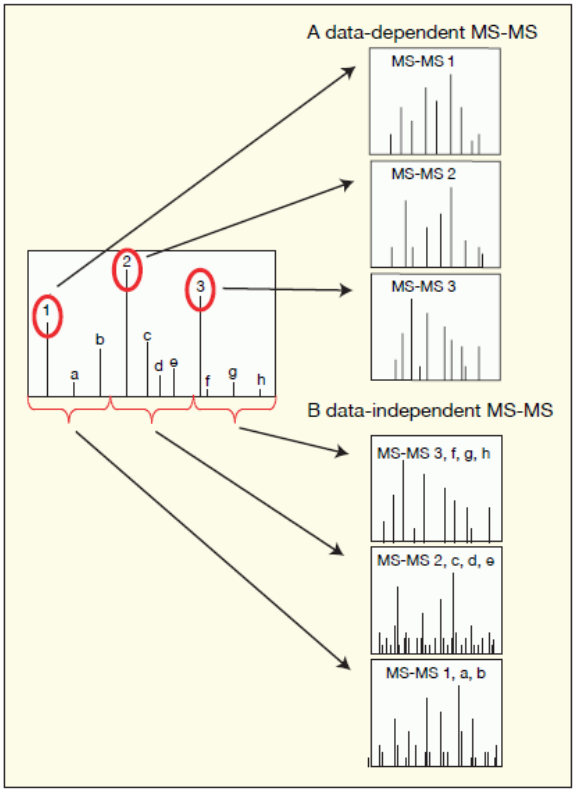


Figure 1.5 Diagram illustrating different data acquisitions. A- Data dependent acquisition whereby the most common peptide ions are selected (1-3). The low abundant ion a-h are not selected and omitted from MS/MS data. B- Data independent acquisition all peptides ions (1-3, a-h) within the mass range are fragmented and are thus analysed (Liebler, 2004)**.**

1.2.10.3 **Quantification**

The quantification of proteins is the essence of biomarker discovery, as the aim is to show differential expression of proteins between disease and disease-free samples. The quantification of proteins can be performed by using either stable isotope labelling or label-free methods. Isotope label quantification is characterised by the incorporation of stable isotope labelled standard peptides. The intensity of the labelled standard is compared with the endogenous unlabelled peptides and their relative abundance is calculated (Kline and Sussman, 2010). This technique is limited by lengthy sample preparation times and the high cost of the reagents. In addition, multiple protein quantification in samples is difficult due to the limited number of isotope labelled reagents available (Wang *et al*, 2008). In contrast, spectral counting is a label-free technique, whereby the relative protein quantification is determined by comparing the number of identified spectra from

the same protein in multiple LC-MS/MS runs (Liu, Sadygov and Yates, 2004; Zhu, Smith and Huang, 2010). Spectral counting has the advantages of being a simpler and faster method (Chen and Yates, 2007). The adoption of spectral counting was justified by Liu who demonstrated that it had the strongest correlation with relative protein quantification (Liu, Sadygov and Yates, 2004). Label-free quantification of ions can also be achieved by data independent MS^E methods, whereby precursor ions are subjected to high and low energy in the collision cell to generate product ions. Quantification can be achieved by measurement of the three most intense peptides (MS1) of each protein, which is then compared to the top three peptides of an internal standard. MS^E functions by the quadrupole directing the peptides to the collision cell with either low or high collision energy rates. The mass of the intact peptides is measured in the low energy state whilst the mass of the fragment ions is measured in the high energy state.

There are a number of bioinformatics software programmes that are available, such as Scaffold, Progenesis and ProteinLynx Global Server (PLGS 2.5, Waters Corporation), that serve to identify and quantify the proteins in the mass spectra produced by the MS.

1.3 **Hypothesis**

The hypothesis for this study is that subtle differences exist in the proteomic cargo of lipoproteins of South Asian patients with CAD, compared to sex matched Caucasian patients, potentially explaining the differential CAD risk.

1.4 **Aims & objectives**

The aims of this project are set out below;

1. To develop a method for lipoprotein isolation, enabling the analysis of their unique low abundant protein cargo, in a bottom-up label free MS approach.
2. To optimise this discovery workflow to enable efficient and reliable lipoproteomic quantification for application in the clinical studies.

3. To determine the remodelling effect of statin therapy on lipoproteins and their associated low abundant protein cargo.
4. To establish the lipoproteomic differences between patients with CAD compared to health control subjects, to identify surrogate markers of disease or identify new mechanism of disease pathogenesis.
5. To determine the lipoproteomic differences between South Asian patients with CAD compared to Caucasian patients with CAD, which may identify novel markers that may account for the excess risk observed in the former ethnicity.

Chapter 2

2 General Materials and Methods

2.1 Materials and Instruments

Lipid removal agent (calcium silicate hydrate) reagents and desalting columns were purchased from Sigma Aldrich (Poole, UK). Tartaric acid and copper sulphate used for bicinchoninic acid protein assay were purchased from Fisons Scientific Apparatus (Loughborough, UK). A Multiple Affinity Removal System (MARS) Human 14 column along with compatible eluting buffers and spin filters were purchased from Agilent Technologies (Stockport, UK). Spin columns with a 3 kDa molecular weight cut-off (Amicon Ultra-0.5 ml Centrifugal Filters) were purchased from Millipore (MA, USA). Mass spectrometry standards, Leucine enkaphalin, Glu-fibrinogen peptide (GFP), alcohol dehydrogenase and analytical columns were purchased from Waters Ltd., (Manchester, UK). Optima LCMS solvents, HeLa protein digest were purchased from Thermo Fisher Scientific Ltd (Loughborough, UK). A Thermo multiscan spectrophotometer was used for measuring light absorbance. A Berthold MicroLumat Plus luminometer from PerinElmer was used for measuring bound chemiluminescence.

2.2 Plasma Samples

Method development was performed on plasma samples donated from healthy volunteers after informed consent. Clinical discovery studies were performed on plasma samples donated from patients after informed consent. The clinical discovery studies were approved by the regional ethics committee (REC reference: 13/EM/0049) and were in accordance with the Declaration of Helsinki. Blood was withdrawn from consented volunteers/patients and collected in pre-chilled 30 mL Universal containers containing 80µL 1M EDTA (Sigma Aldrich) in 2M NaOH & 250 µL Trasylol (Nordic Pharma [Reading, UK]) per 20 mL of blood. Samples were centrifuged at 1500 G for 15minutes at 4°C, the plasma was aliquoted into eppendorfs and stored at -80 °C until analysis.

2.3 Lipoprotein Isolation

LRA (Sigma Aldrich [Poole, UK]) is predominantly composed of silica (56.78%) and calcium oxide (37.38%), Table 2.1. LRA is extremely insoluble in water and is weakly basic with a 10 g/L solution giving a pH of 8.7.

Table 2.1 Chemical composition of LRA

Chemical composition	Content (%)
SiO_2	57.78
CaO	37.38
Al_2O_3	2.82
Fe_2O_3	0.93
MgO	0.8
K_2O	0.33
Na_2O	0.26
P_2O_5	0.23
SO_3	0.21
TiO_2	0.13

In this study, the binding kinetics of LRA for lipids and lipoproteins was extensively explored to identify a novel method for assessing the proteomic cargo of lipoproteins.

2.4 Proteomic workflow

2.4.1 Bicinchoninic acid protein assay

The basis of the bicinchoninic acid (BCA) protein assay is a colour dependent reaction between the copper (Cu 2+) ions and the peptide bonds. The resulting solution changes from blue to purple, a consequence of the reduction of the copper ions. The colour absorbency is directly related to the protein concentration and is measured on a spectrophotometer (wavelength 562 nm). The generation of a

stable copper protein complex in an alkaline condition is resistant to the effects of detergents and therefore adds to its versatility, Figure 2.1.

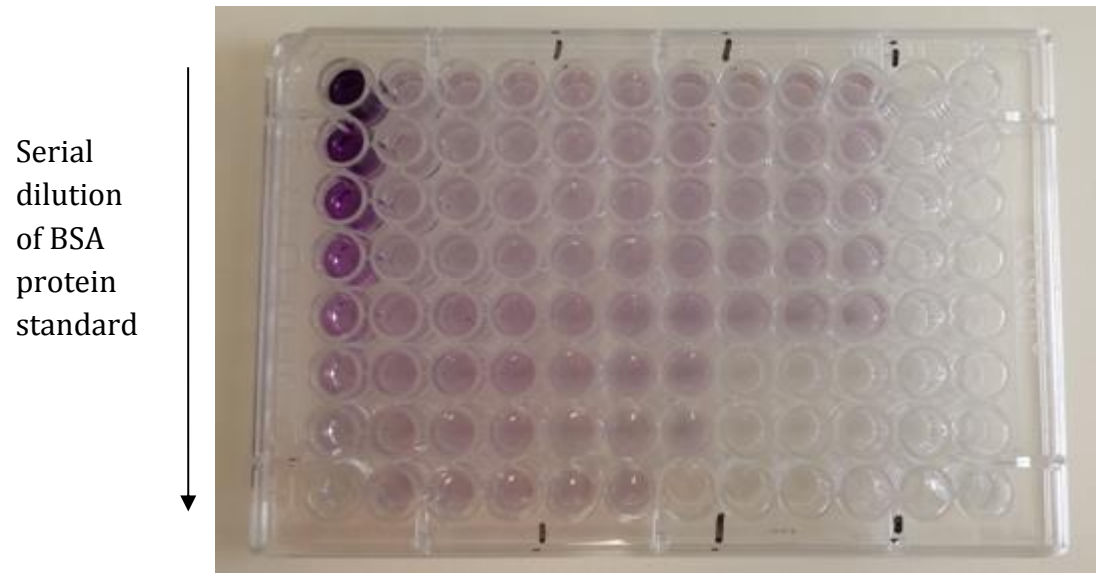


Figure 2.1 BCA assay. The top well contains 80 µg of BSA and is serially diluted 2-fold; hence the decreasing colour intensity signifies a decreasing protein concentration.

2.4.1.1 **Reagents**

Solution A: 8 g sodium carbonate monohydrate, 1.6 g Tartaric acid (Fisons Scientific Apparatus) 100 mL of water, pH 11.25

Solution B: 1 g BCA in 25ml water

Solution C: 0.4 g copper sulphate (Fisons Scientific Apparatus) in 10ml water

Working solution: 50 µL of solution C, 1.25mL of solution B and 1.35 mL of solution A

2.4.1.2 **Procedure**

The first column of the microtitre plate was used for the standards which comprised of serial dilutions of bovine serum albumin (BSA). 120 µL of deionised water was added to the first well, with 100 µL added to the remaining wells. 80 µl

(80 µg) of BSA was pipetted into the first well, followed by a 2-fold serial dilution in the subsequent wells. The final well comprised of water alone and represented the zero of the standard curve. The top standard represented 40 µg of BSA. 100 µl of the reagent mix was added to all wells for the reaction to occur.

For the quantification of the protein content in samples, 1 µL of sample was incubated with 100 µL of deionised water, followed by the addition of 100 µL of the regent mix. The microtitre plate was subsequently covered to prevent evaporation and incubated at 60 °C for 30 minutes. Samples were run in duplicate. The light absorbance was measured on a Thermo Multiscan spectrophotometer. The light absorbance on the standard curve was then used to calculate the protein concentrations of the samples. A typical standard curve is shown in Figure 2.2.

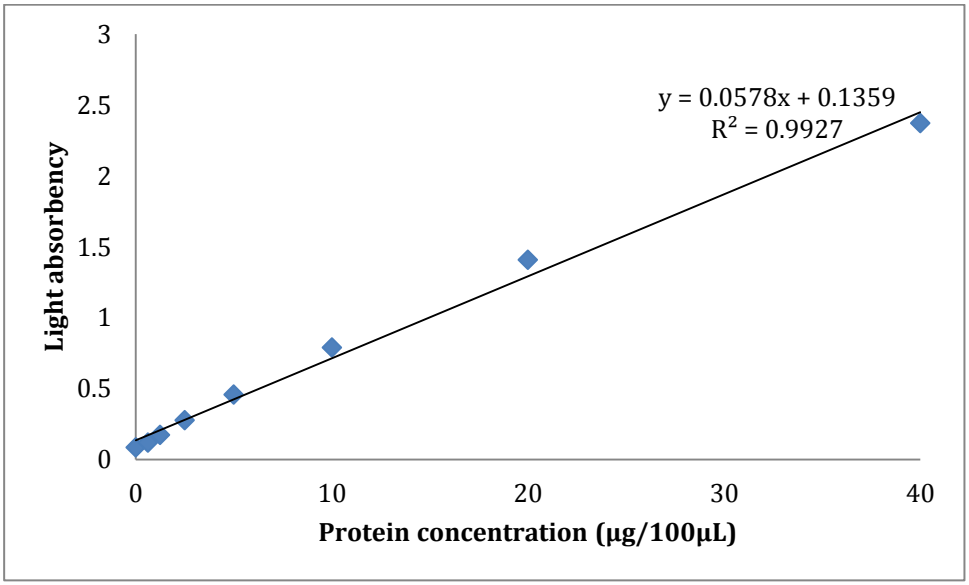


Figure 2.2 Standard curve for BCA protein assay

2.4.2 Multiple Affinity Removal System

The presence of high abundant proteins in the samples can hamper the discovery of low abundant proteins. A number of commercial columns are available that specifically target and remove the high abundant proteins, decreasing the dynamic range of the sample and facilitating the discovery of low abundant proteins. The multi affinity removal system (MARS, Agilent Technologies) removes the top 14 high abundant proteins from the sample (Table 2.2). The MARS column is coupled to HPLC. The column contains antibodies with affinity for the top 14 proteins. As

the sample passes through, the high abundant proteins become bound, while the low abundant proteins flow through and are collected. The interaction between the low abundant proteins and the high abundant proteins is minimised by the use of a neutral salt buffer, that the manufacturer supplies. A urea containing buffer is then used to disrupt the interactions between the antibody-antigen complexes, enabling the collection of the high abundant proteins in the flow through. The collected samples are then concentrated, using spin filers and buffer exchanged. The protocol that was used is described below.

Table 2.2 Top 14 high abundant proteins removed by MARS

	Proteins
1	Albumin
2	IgG
3	IgM
4	IgA
5	Haptoglobin
6	Fibrinogen
7	Transferrin
8	Alpha1- antitrypsin
9	Alpha 2-macroglobulin
10	Complement C3
11	Alpha 1-Acid glycoprotein
12	ApoA-l
13	Transthyretin
14	ApoA-ll

2.4.2.1 **MARS Protocol**

- Samples were initially diluted in buffer A (Agilent Technologies) to make a final volume of 250 µL.
- HPLC was equilibrated prior to sample run, with the UV wavelength set at 280 nm.
- 200 µL of the samples were injected into the MARS column (Agilent Technologies).
- The low abundant proteins were typically collected from 10-15 minutes, with the high abundant proteins typically eluted at 22 minutes.

- Protein fractions were concentrated using Amicon spin filters (Millipore) and spun for 15 minutes to remove proteins below 5 KDa.
- Proteins were subsequently buffer exchanged with 50 mM ammonium bicarbonate (AmBic) to remove salts that interfere with MS analysis.

2.4.3 Digestion

The process of protein digestion into peptides relies upon the disruption to the quaternary and tertiary structure of proteins. Dithiothreitol (DTT) is a powerful reducing agent that reduces the disulphide bond between cysteine residues in the proteins. A final concentration of 25 mM DTT (Sigma Aldrich) was added to the samples and was incubated at 60 ^{o}C for 30 minutes. Iodoacetamide (IAA) is a potent alkylating agent that is commonly used in the digestion process. IAA reacts with free thiol groups on cysteine residues to form s-carboxylmethylated cysteines, thus preventing the reformation of the disulphide bonds. Typically, a final concentration of 20 mM IAA (Sigma Aldrich) was incubated with the sample in the dark at room temperature for 30 minutes. There are various digestion agents available such as pepsin and chymotrypsin, but the most commonly used in proteomics is trypsin, due to generation of small fragments, which are ideal for LC-MS/MS analysis. This serine protease cleaves the C-terminal of lysine and arginine residues except when they are followed by proline. The trypsin used in proteomics is acetylated to prevent autolysis and eventual signal suppression. The protein concentrations derived from the BCA protein assay were then used to guide the amount of trypsin to be added to each sample. Typically for every 25 µg of protein in the sample, 1 µg of trypsin (Waters Ltd) was added. Samples were incubated overnight at 37 °C.

2.4.4 Solid phase extraction

Solid phase extraction (SPE) a preparative technique used to purify samples prior to analysis. In proteomics, SPE is used to purify the protein content of samples and to eliminate contaminating interferences such as small molecules or detergents. Empore C18 cartridges were employed as a clean-up process for desalting and

concentrating the samples. The empore C18 cartridges contain a disc of sorbent fibres of varying densities along with silica sorbents. The analytes of interest pass through the fibres and become trapped by a combination of the meshwork and the hydrophobic interactions exerted by the octadecyl hydrocarbons.

2.4.4.1 **Procedure**

Prior to SPE, the digested samples were acidified to a final concentration of 1 % formic acid (FA) to halt protein digestion. The Empore C18 4 mm/1 mL catridges (Sigma Aldrich) cartridges were conditioned with 1ml of methanol and subsequently washed three times with 1ml 0.1 % FA. The sample was placed onto the Empore C18 catridges and the flow through was re-circulated to enhance peptide recovery. The cartridges were subsequently washed three times with 1 mL of 0.1 % FA to remove any impurities. The trapped peptides were eluted using varying concentrations of organic solvent, typically with 500 µL of 60 % acetonitrile (ACN) followed by 500 µL of 80 % ACN into eppendorfs. The samples were concentrated in the SpeedVac concentrator for 2 hours at 50 ˚C and lyophilised overnight in a freeze dryer.

2.4.5 Internal standard

The lyophilised samples are re-suspended in 0.1 % FA. For label free quantification, the samples were spiked with an equal volume of internal standard comprising of alcohol dehydrogenase (ADH, Waters Ltd.). Typically, 50 femtomols of ADH was injected onto the column for quantification (Silva *et al*, 2006).

2.4.6 LC-MS analysis

Methods were developed using the Thermo Q Exactive (Thermo Scientific, Bremen, Germany) which was coupled to an Ultimate 3000 RSLC nano HPLC system (Dionex/Thermo Fisher Scientific, Bremen, Germany). The samples were preconcentrated by the 300 µm x 5 mm C18 PepMap (5 µm, 100A) trap column and then separated by a 75 µm x 50 cm Easy-Spray pepMap C18 analytical column.

The tryptic peptides were eluted from the C18 column over a 75 minute gradient of aqueous mobile phase A (0.1 % FA in water) and organic mobile phase B (80 % ACN, 0.1 % FA in water). The eluting gradient comprised of an isocratic concentration of 3% organic mobile phase B for 3 minutes to condition the column followed by ramp up to 10 % at 3.2 minutes. The gradient of organic mobile phase B increased linearly to 50 % from 3.2 to 40 minutes to elute the majority of the peptides. A steep gradient of 50 % to 90 % of organic mobile phase B occurred between 40 to 45 minutes to elute the remaining hydrophobic peptides and was maintained at 90 % for 5minutes. The column was subsequently equilibrated with 3 % organic phase B for the remainder of the injection. The eluting gradient of organic mobile phase B is presented below in Figure 2.3. The flow rate was maintained at a rate of 0.3 µL/min. The analytical C18 column was controlled at 40 ˚C and the autosampler was maintained at 8 ˚C.

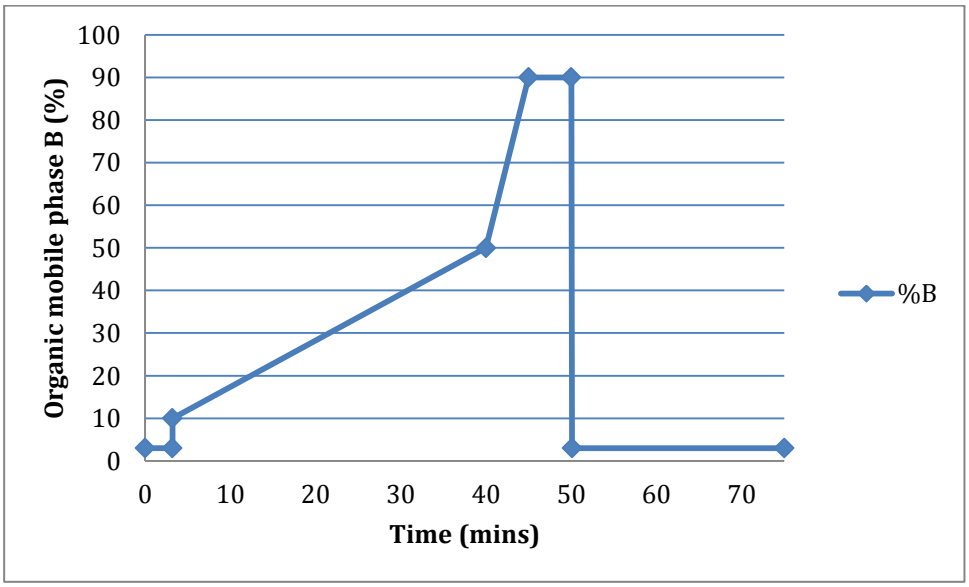


Figure 2.3 Eluting gradient of buffer B

2.4.7 Q-Exactive Orbitrap

The Thermo Q exactive (Thermo Scientific [Bremen, Germany]) was operated in data dependent top 10 mode. For the survey scans the most abundant parent ions were chosen with a resolution of 70000 with m/z of 200-2000 for fragmentation. For this survey scan the target number of ions that was injected into the analyser was set to an automatic gain control (AGC) of $1e^6$ with the scan time limited to a

maximum of 50 ms. The product ions were then acquired with a resolution of 17500. The AGC for MS/MS was set to a target of $1e^5$ with a predefined time limit of 100 ms if the AGC was not reached.

2.4.8 Quality Control for MS analysis

HeLa protein digest (Thermo Fisher Scientific Ltd) was used as a quality control for MS analysis.

- HeLa protein digest (20 µg) was diluted with 20 µL of 0.1 % of FA
- 1 µg was loaded onto the analytical column to achieve 2000 protein hits to ensure optimal performance of the MS.

2.4.9 Data processing

The Thermo raw files were analysed on Thermo Proteome Discoverer 1.4, which used the Uniprot human database (downloaded November 2013) for protein identification. Carbamidomethylation of the cysteine residues were set as fixed modifications, along with the oxidation of the methionine residues as variable/dynamic modifications. All analyses were performed using a false discovery rate (FDR) of 1 %.

2.4.10 Waters Synapt G2S

2.4.10.1 **LC conditions**

The clinical studies were analysed on a Waters Synapt G2S (Waters Ltd.). This MS was coupled to a nanoAcquity UPLC with a 5 µm symmetry C18 trapping column followed by a 1.8 µm HSS T3 75 µm x 150 mm analytical column for peptide separation. The role of the trapping column was to concentrate and desalt the sample, whilst chromatographically focussing the peptides prior to the elution to the analytical column. The aqueous mobile phase A comprised of 0.1 % FA and 5 % DMSO. The organic mobile phase B comprised of 0.1 % FA in ACN and 5 % DMSO. An increasing gradient of mobile phase B was used to elute the peptides,

depending on their hydrophobicity, from the analytical column at a flow rate of 0.3 µL/min over 110 minutes. Typically, the analytical column was conditioned with 1 % organic mobile phase B for the first 2 minutes followed by a linear gradient reaching 40 % organic mobile phase B by 60minutes, to elute the majority of the peptides. Subsequently a steeper gradient of organic mobile phase B ensued reaching 85 % by 80 minutes, which was then maintained for 5 minutes, with the aim of eluting the remaining hydrophobic proteins. The column was then equilibrated at 1 % organic mobile phase B for the remainder of the injection. The pattern for organic mobile phase B over the injection is presented in Figure 2.4. The column temperature was set to 40 °C and the auto sampler was maintained at 8 °C.

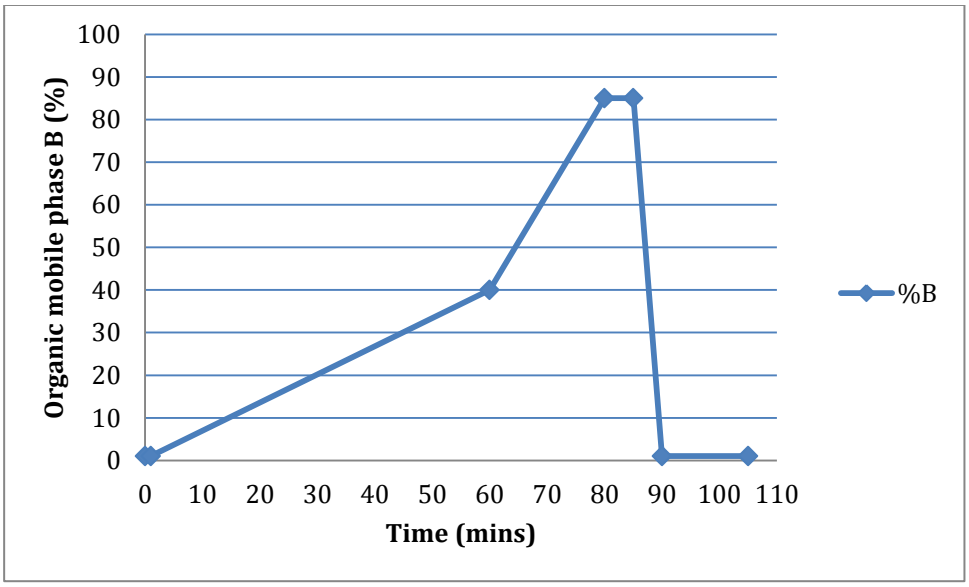


Figure 2.4 Gradient for mobile phase B

2.4.10.2 **MS conditions**

The Waters G2S MS was operated in positive ESI mode with the capillary voltage set to 2.8 kV and a source temperature of 80 ˚C. Label free data independent acquisition (DIA) was carried out using alternate scans at low and high energy (MS^E). Data acquisition occurred over a mass range of 50-2000 m/z. The scan time for each mode was 1.0 second. The data were acquired in low energy mode with

the collision energies maintained at 4-6 eV and the high energy data were acquired with ramps in collision energies of 20-45 eV. When ion mobility was utilised this experiment is referred to as high definition MS^E ($HDMS^E$).

Three preliminary checks were performed to ensure instrument performance and consistency; detector setup, mass calibration and lock mass source setup. The sensitivity of the detector was assessed using Leucine enkephaline (Leu-Enk) m/z 556.2, which was infused via a nanostage holding a PicoTip emitter 10µm (New Objective, USA). [Glu^1] fibrinopeptide β (GFP) was used for calibration of the MS. This was delivered to the reference emitter at a flow rate of 0.5 µL/min. Successful calibration was dependent on the identification and matching of 15 measured ions to reference ions for GFP. In the event of calibration failure, the lock mass sprayer was adjusted and recalibrated. In addition, GFP was used as the lock mass channel, which sampled every 30 seconds during a sample injection. Data independent acquisition was performed enabling the simultaneous collection of data for both parent and product ions at no additional expense of analytical time. When ion mobility capability was enabled the wave velocity and wave height were set to 65 m/s and 40 V respectively. Data were acquired and analysed using Mass Lynx version 4.1.

2.4.11 Data processing

2.4.11.1 **Protein identification**

2.4.11.1.1 Protein Lynx Global Server

Protein Lynx Global Server (PLGS) 3.0 was used for protein identification of the G2S raw data. The identification process is complex and uses various iterative steps. In brief, this processing relies upon the physicochemical attributes of the parent and product ions, which are scored and ranked. Once respective product ions are matched to parent ions they are removed from the original ion pool, with the remaining ions undergoing further processing. The matched product and parent ions are searched against tryptically generated peptide sequence databases. The strength of this programme is the incorporation of decoy or dummy proteins and their respective amino acid sequences, with their selection

leading to the user defined false discovery rate. The specificity of ion selection for protein identification is enhanced by the use of additional ion attributes. (Li *et al*, 2009).

2.4.11.1.2 Scaffold

Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to for comparing the protein hits between the different experimental methods. The MS raw files were analysed using PLGS with a FDR of 100% and exported into the Scaffold software. The advantage of the Scaffold software is that is offers greater reproducibility and confidence in peptide and protein identification. In brief, the software initially performs several open source or vendor specific databases search enquiries for peptide identification such as SEQUEST and Mascot (Eng, McCormack and Yates, 1994; Perkins *et al*, 1999). The peptide scores are combined and verified using X!Tandem search database (Craig and Beavis, 2003). This improves confidence in peptide identification. The peptide probability scores are then analysed using a Protein Prophet algorithms and assigned into protein groups (Searle, 2010).

2.4.11.2 **Expression analysis**

Progenesis Qi for proteomics was used for analysing differential protein expression between the different experimental groups. Progenesis QI enable analysis of label free data by employing several steps:

Peak modelling

The data files that are generated from the LC-MS/MS runs are very rich in spectra and size. Progenesis Qi uses peak modelling to compress the size of the files. This is achieved by removing the signal from the background noise and by simplifying the peaks using peak modelling algorithms. The sizes of the files are drastically reduced from gigabytes to megabytes without the intrinsic loss of the data (Nonlinear Dynamics, 2015).

Alignment

Over a number of samples runs, especially in large datasets, there can be drift in the retention times for the ions, which may lead to under sampling of ions and ultimately reduced protein identification and quantification. Progenesis Qi aligns the sample runs by matching the retention times of the ions to the references ions, enhancing protein identification. The reference run can be automatically chosen by analysing the suitability of all runs in the dataset or from a preselected group of runs. The preselected runs tend to ideally consist of pooled sample runs, which are more representative of the entire cohort. The software then creates vectors, which link the ions in the sample to the reference ions in the reference run. This is performed for all samples. The alignment for the sample runs is then scored against the reference run, to indicate consistency of alignment and highlights any samples which need review(Nonlinear Dynamics, 2015).

Peak Picking

The peaks from all the sample runs are grouped into a single peptide ion map. This ensures that the total numbers of ions identified in peak picking are individually checked in each sample, increasing reliability and improving peptide identification (Nonlinear Dynamics, 2015).

Ion abundance quantification

Progenesis Qi quantifies the data first before identification. Precursor ions that are differentially expressed between the experimental groups are subsequently database searched this avoids potential loss from the low abundant ions that may not have MS/MS data. This feature of progenesis Qi is attractive as the low abundant ions tend to be desirable in biomarker discovery. The ions are then normalised to adjust for differences in volume injection etc. A scalar factor is calculated and applied to all samples normalising to all proteins, unlike normalising to the total ion intensity which is heavily biased towards the high abundant proteins (Nonlinear Dynamics, 2015).

Protein identification

The peptide ions are then searched against a human database. Proteins can be quantified absolutely if an internal standard such as ADH has been used consistently in each run, and is present in the same concentration. However, when the volumes of the samples have been adjusted to ensure adequate and consistent protein loading between samples, relative quantification (Hi-N) is used. In this case the most abundant peptide ions (N) for each of the proteins is averaged and applied to each protein in each sample for relative quantification. Various statistical tests such as ANOVA and power analysis are performed between the experimental conditions and the differentially expressed proteins are then outputted (Nonlinear Dynamics, 2015).

2.4.11.3 **Pathway analysis**

Pathway analyses were performed using an open source bioinformatics tool. The interaction between the various differentially expressed proteins was assessed using the Search Tool for the Retrieval of Interacting Genes/proteins (STRING) version 10 (Szklarczyk *et al*, 2015). This resource provides functional properties of the proteins and gives a greater insight into how such proteins cooperate to mediate protective or deleterious actions in disease/health. The methods for prediction of the protein-protein interaction utilised information from the following;

- Neighbourhood; genes located in close proximity
- Gene fusion; genes that are known to fuse in different species
- Co-occurrence; proteins that are linked across different species
- Co-expression; genes which are expressed in the same species or different species
- Experimental data; information relating to protein interaction derived from alternative databases
- Databases; protein interaction from curated databases
- Text mining; information relating to protein interaction derived from abstracts and scientific texts (Szklarczyk *et al*, 2015).

Cluster analysis was performed to delineate salient protein interaction clustering (Szklarczyk *et al*, 2015).

2.4.12 Immunoassays

Immunoassays are analytical techniques that use antibodies for the capture, detection and quantification of proteins/antigens in various samples ranging from plasma to urine. The high specificity afforded by antibodies enables the detection of specific antigens, even in the presence of similarly related species. Their versatile nature also enables their immobilisation to various surfaces such as plastics. Furthermore, the antigen-antibody complex is resistant to processing steps due to the strong interactions. Immunoassays have evolved from their inception back in the 1960's to become the cornerstone for analyte quantification. Detection is based on the use of various chemical tags, which either emit radiation, fluorescence or chemiluminescence, on exposure to the antibody-antigen complex. Immunoassays can be categorised into competitive and non-competitive designs.

2.4.12.1 **Competitive immunoassays**

Competitive immunoassays are principally used when the analyte in question is too small to accommodate the binding of two antibodies. A monoclonal antibody is used to bind to a specific epitope on the antigen, which is referred as the capture antibody. A known amount of labelled analyte competes with analyte for the binding sites on the capture antibody. The signal generated is inversely proportional to the amount of analyte in the sample.

2.4.12.2 **Non-competitive immunoassays**

The capture antibody is immobilised on a plastic surface and serves to capture the analyte in the sample. Samples are incubated along with calibrators for the generation of a standard curve. A second antibody referred to as the detection antibody, is labelled with biotin and binds to a specific epitope on the antigen,

effectively sandwiching the analyte. The biotintylated antibody binds avidly to streptavidin creating a chemiluminescent tag. Bound chemiluminescence is subsequently measured and is directly related to the amount of analyte in the sample. The basis of this immunoassay design was used in this study for quantification of apoA1.

2.4.12.3 **ApoA1 Immunoassay**

The instructions for the apoA1 immunoassay were refined from the manufacturer's protocol to improve signal detection. The protocol was as follows;

- 100 ng of apoA1 rabbit polyclonal antibody (Sigma Aldrich) was used to coat the wells of the 96 well microtitre plate.
- Microplates were incubated overnight at room temperature to enable immobilisation of the capture antibody to the plastic surface of the well.
- Plates were washed with phosphate buffered solution (PBS) to remove unbound capture antibody.
- 150 µL of BSA was added to each well in order to block all potential protein binding sites on the microplates thereby reducing background interference.
- Plates were incubated for two hours at room temperature
- Plates were washed with detergent (wash B)
- Human recombinant apoA1 (Sigma Aldrich) was used as calibrators for the generation of the standard curve, Figure 2.5.
- Top standard consisted of 100 ng of apoA1.
- Unknowns were diluted with ILMA [1.5 mM NaH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 1.0 mM EDTA,1 g BSA, 0.1 g Azide, 0.1 % Triton / 1 L ILMA), pipetted into the wells and incubated overnight.
- Plates were washed with wash B.
- 10 ng of anti-human apoA1 biotinylated goat antibody (Sigma Aldrich) in 1 % goat's serum was added to each well.

Different host species were used to reduce cross reactivity. Goat's serum was used as another blocking agent to reduce the background interference and to improve the signal to noise ratio.

- Microplates were incubated for 4 hours
- Plates were washed in wash B
- 100 µL of Streptavidin was added to each well and incubated for 90 minutes at room temperature in the dark.
- Plates were washed twice with wash B.
- Bound chemiluminescence was measured on a luminometer (Berthold, Germany) Figure 2.5.

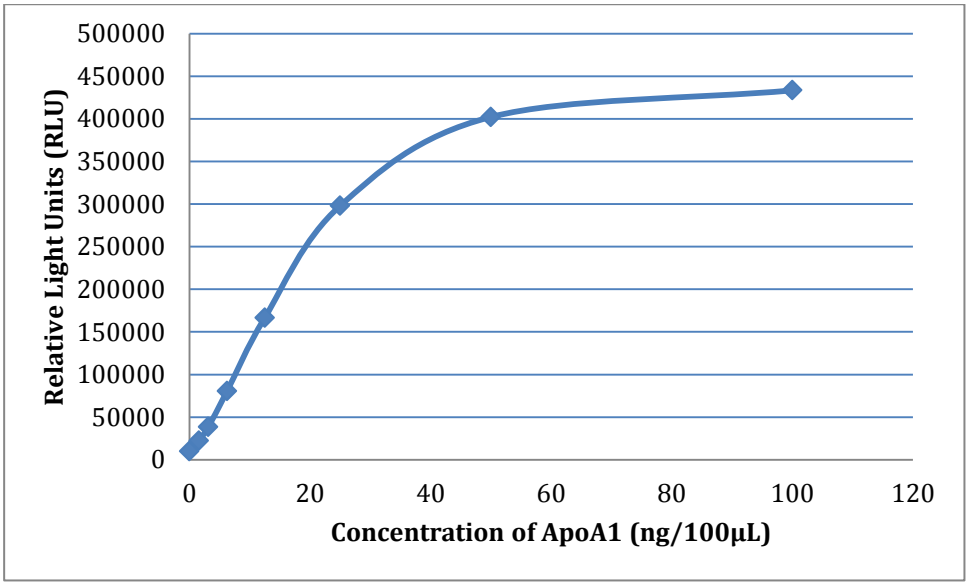


Figure 2.5 Typical Standard curve for the ApoA-1 sandwich assay. The sigmoidal curve indicates that the assay signal becomes saturated with ApoA1 concentrations above 40 ng/100µL. Hence dilution of the ApoA1 standards/samples is required for optimal detection and avoid signal saturation.

Chapter 3

3 Method Development

3.1 Introduction

Critical assessment of the different lipoprotein fractions and their associated proteomic cargo still relies on sequential density ultracentrifugation techniques (Havel, Eder and Bragdon, 1955). The use of such techniques is fraught with technical challenges. The high centrifugal forces used to sediment the fraction can potentially strip the exchangeable proteins from their respective lipoprotein fraction and lead to an underestimation of the true cargo. Furthermore, the use of KBr for generation of the gradients is potentially chaotropic, which could alter the proteome. Novel methods have been developed that exploit different physicochemical features and avoid the pitfalls associated with centrifugation. The use of calcium silicate hydrate also known as LRA has been used commercially to remove lipid from biopharmaceutical products. The use of this resin as a lipoaffinity resin has been previously explored by Gordon et al, whereby the already isolated fractions were co-incubated with this resin and then subjected to the typical proteomic protocol for MS analysis (Gordon *et al*, 2010; Gordon *et al*, 2013). In this chapter, the lipoaffinity characteristics of this resin were explored either in isolation or as an adjunct to other separation techniques. The purpose of this work was to develop a novel method for isolating the lipoproteins and their associated protein cargo, in an unbiased manner that facilitates MS analysis. Initially, the binding properties of the lipoaffinity resin were investigated by measuring the apoA1 concentrations in plasma prior to and after co-incubation with the resin. ApoA1 was used as a surrogate for HDL binding, as it is the main apolipoprotein in this particle. Subsequent experiments focussed on the assessment of the lipoproteomic cargo bound to the resin, with interventions aimed at reducing non-specific binding and the selective extraction of the lipoprotein associated proteins from the resin.

3.2 Aims and objectives

The aims for this chapter were as follows;

1. To develop a reliable and efficient method for isolating the lipoproteins and their associated protein cargos.
2. To optimise this methodology by assessing the influence of pH, cations detergents, solvents on binding affinity of this novel resin.
3. To optimise this workflow for lipoproteomic discovery by assessing the impact of pre-washing, detergents, post-washing, solvents, temperature and desaltation on protein coverage.

3.3 Materials and Methods

3.3.1 Materials and reagents

All materials and reagents were purchased from Sigma-Aldrich, Agilent Technologies, Millipore, Waters Corporation and Fisher Scientific as described in Chapter 2.

3.3.2 Optimisation of the affinity resin

3.3.2.1 Time profile

The efficiency of lipid binding to affinity resin was assessed by determining the apoA1 concentration in the plasma prior to and after co-incubation with the affinity resin. ApoA1 is the main structural scaffold in HDL, therefore quantification of apoA1 was used as a surrogate for the HDL binding to the affinity resin.

- Stock consisting of 100 mg of LRA (Sigma Aldrich) in 9 mL of 25 mM AmBic (Sigma Aldrich) was prepared.
- 900 µL of the LRA stock was added to 100µl of control plasma (LRA 10 mg/mL)

- Samples were incubated with agitation at room temperature for 0, 2, 4, 6 and 24 hours.
- LRA was pelleted by centrifugation (14000 g for 5 minutes) at the different time points and the supernatant was removed.
- Supernatant was diluted 500-fold with ILMA.
- 100 µL of diluted supernatant were assayed for determination of the apoA1 concentration using the assay design described previously in 2.4.12.3.

The affinity resin was efficient in depleting apoA1 from the plasma, Table 3.1. Within 2 hours of plasma incubation, apoA1 levels were depleted by nearly 90%, suggesting that this method isolated the lipoproteins, in particular HDL, in a timely fashion, compared with conventional techniques such as sequential density ultracentrifugation. Shorter co-incubation periods between the affinity resin and plasma were examined further.

Table 3.1 Time course (over 24h) showing the removal of apoA1 from plasma co-incubation with the affinity resin (LRA 10 mg/mL), n=1.

Time (hours)	ApoA-1 concentration in plasma incubated with affinity resin (mg/dL)
0	93.61
0.16	86.74
2	9.56
4	9.57
6	8.54
24	7.13

3.3.2.2 **Modified Time Course of apoA1 depletion from plasma using LRA**

In view of the rapid depletion of lipoproteins, in particular apoA1, by the affinity resin we sought to investigate the effect of shorter plasma incubation periods. Shorter incubation periods would drastically improve throughput. The experiment was repeated as in 3.3.2.1 except for the doubling of the concentration of the affinity resin to 20 mg/mL.

Concentrations of apoA1 were rapidlydepleted from the plasma Table 3.2. Within 30 minutes of plasma co-incubation with the affinity resin, apoA1 was completely adsorbed onto the resin, owing to a higher concentration of affinity resin compared with the previous experiment. The lipophilic properties of this agent have been utilised as a clean-up step for biopharmaceutical products (Zhang *et al*, 2005). With this feature in mind, such a characteristic would prove useful for the isolation of lipoproteins and their associated proteins for proteomic discovery. The use of such a lipoaffinity resin, in preference to conventional sequential density ultracentrifugation, avoids the disadvantages such as protein desorption, from the high centrifugal forces and the chaotropic effects from KBr. Furthermore, the isolation of the lipoproteins in a timely fashion would enable high throughput of clinical samples, which contrasts with the long centrifugal times, required for the fractions to sediment in sequential density ultracentrifugation, especially when the distinction between the lipoprotein fractions is unnecessary for this study.

Table 3.2 Extended time profile study (0-240min) of apoA1 concentrations in plasma in the presence of the LRA (20 mg/mL), detected by ELISA. Data values expressed as mean ± standard deviation (n=3). ApoA1 concentrations diminished rapidly with increasing co-incubation times.

Time (minutes)	ApoA-1 concentration in plasma incubated with the affinity resin (mg/dL)
0	39.18 ± 8.57
5	8.00 ± 0.36
15	0.90 ± 0.55
30	0
45	0
60	0
120	0
240	0

3.3.2.3 **Determination of optimal LRA concentration**

The optimal concentration of the affinity resin was investigated. Plasma was co-incubated with increasing concentrations of the affinity resin, which ranged from 10 - 100 mg/mL and apoA1 concentrations were determined after 2 hours of plasma incubation. The design of the experiment was similar to that described in 3.3.2.1.

Table 3.3 shows that using a 10 mg/mL concentration of the affinity resin removed almost all apoA1 from plasma. Complete removal of apoA1 was achieved with concentrations greater than 10 mg/mL. Concentrations of ≥10 mg/mL were used in subsequent experiments to ensure adequate adsorptive capacity.

Table 3.3 ApoA1 concentrations in plasma after co-incubation with increasing concentrations of the affinity resin (0-100 mg/mL), detected by ELISA, n=1.

Concentration of affinity resin media (mg/mL)	ApoA-1 concentration in plasma incubated with the affinity resin after 2hours (mg/dL)
0	96.74
10	1.09
20	0
50	0
100	0

3.3.3 Binding modification

3.3.3.1 Influence of pH on the lipid binding capacity of LRA

The effect of altering the pH of the affinity resin media on lipid binding was investigated. The pH range examined was between pH 4 and 8; the extremes in pH were avoided because of the strong possibility of protein denaturation and the risk of dissolving the affinity resin.

- Stock solution of 10 mM sodium acetate (Sigma Aldrich) was prepared and further acidified with 10 mM acetic acid to attain pH 4-6.
- Stock solution of 25 mM AmBic (Sigma Aldrich) was prepared and acidified with 10mM acetic acid to attain a pH 7.
- Stock solution of 25 mM AmBic was made alkali (pH 8) with 10mM Tris solution (Sigma Aldrich).
- 100 µL of plasma was added to 900 µl of the acidic/basic solution
- 20 mg of LRA was added to solutions.
- Samples were agitated at room temperature for 15 and 120 minutes.

- LRA was pelleted by centrifugation (14000 g for 5 minutes) at 15 minutes and 120 minutes time points and the supernatant was removed.
- Supernatant was diluted 500-fold with ILMA and 100 µL was assayed for ApoA1 concentration as described in 2.4.12.3.

The lowest limit of detection for the apoA1 assay was 8977.95 relative light units (RLU) Figure 3.1. The signals generated for the various affinity resin media were below the limit of detection, indicating complete removal of apoA1 from plasma. The effect of an acidic or basic environment had little effect on the binding affinity of the resin for apoA1. The results suggest that the lipid binding affinity of the resin was unaffected by changes in pH, Table 3.4.

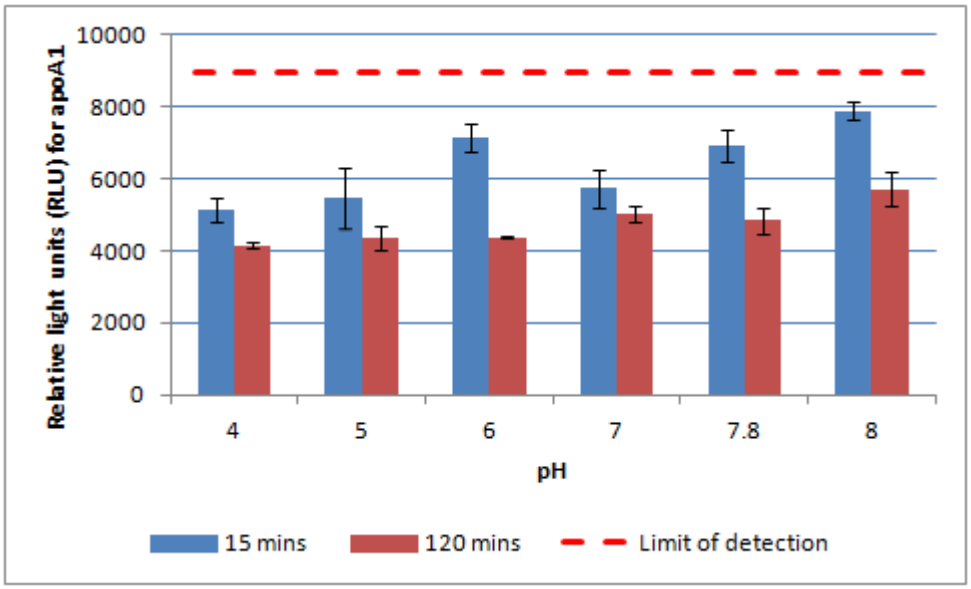


Figure 3.1 The effect of pH on incubation with two time points of LRA with ApoA1. Bar charts showing apoA1 chemiluminescent signal in the plasma samples co-incubated with 20 mg/mL of LRA of different pH measured after 15minutes and 120minutes incubation times. Values are expressed as mean ± SD (n=3)

Table 3.4 ApoA1 concentrations in plasma co-incubated with LRA (20 mg/mL) of differing pH (pH 4-8), detected by ELISA (n=3).

Sample	ApoA-1 concentration (mg/dL)
Plasma control	39.19 ± 8.57
LRA media;	
pH4	0
pH5	0
pH6	0
pH7	0
pH 7.8	0
pH 8	0

3.3.3.2 Influence of cations on lipid binding to LRA

The effect of cations and the non-ionic non-denaturing detergent on the binding affinity of the resin for apoA1 was investigated. Cations such as Mg^{2+}, Ca^{2+} and Al^{3+} may influence protein-protein interactions and therefore alter their binding affinity to the lipo-affinity resin. Proteins and lipids associate via non-covalent hydrophobic interactions, forming micelles. It was hypothesised that the incorporation of cations or the non-ionic non-denaturing detergent to the resin media would alter the ionisation of the amino acid residues and the phosphate groups of the lipoproteins/phospholipids respectively and would therefore impair the adsorptive capacity of the resin for apoA1.

- 1 mM solutions of magnesium chloride/ calcium chloride/ aluminium chloride hexahydrate (Sigma Aldrich) in 25 mM AmBic were prepared.
- 1% NP40 in 25 mM AmBic was prepared.
- 100 µL of plasma were added to 900 µl of the cation/detergent solutions (Sigma Aldrich).
- 20 mg of LRA was incubated with the sample for 15 and 120 minutes.
- The remainder of the protocol is similar to that described previously in section 3.3.2.1.

Table 3.5 indicates that the co-incubation with cations had a negligible effect on apoA1 binding to the affinity resin. This suggests that the resin is extremely robust to changes in the pH and to the presence of cations and detergent nonidet P40 (NP40). Such properties are desirable and support its use as a platform in which to explore the proteomics cargo of lipoproteins.

Table 3.5 ApoA1 concentrations in plasma co-incubated with 20 mg/mL of affinity resin in the presence of magnesium ions, calcium ions, aluminium ions and NP40, measured after 15 and 120 minutes incubations times, detected by ELISA. Values are expressed as mean ± standard deviation (n=3).

Sample	ApoA-1 concentration (mg/dL) at 15mins	ApoA-1 concentration (mg/dL) at 120mins
Plasma Control		56.83 ±11.40
Plasma co-incubated with LRA	0.94 ± 0.61	0
Plasma co-incubated with LRA in the presence of :		
Magnesium	0	0
Calcium	1.11 ± 0.30	0
Aluminium	1.66 ± 0.86	0.68 ± 0.77
NP40	0.10 ± 0.09	1.35 ± 0.47

3.3.3.3 The influence of solvents on lipid binding to the affinity resin

Organic solvents have been previously used to extract lipids from plasma. In this experiment chloroform (Fisher Scientific) and tetrachloroethylene (Sigma Aldrich) were used on the premise that the lipoproteins and their micelles would be disrupted to a greater extent and bring more of the apolipoproteins into solution. Therefore, apoA1 concentrations were determined in plasma that was pre-treated with choloroform and tetrachloroethylene prior to co-incubation with the affinity resin.

Equal volumes of solvent were diluted with water. The purpose of this was to saturate the solvent and to prevent possible water loss from the plasma when mixed with the solvent.

- 300 µL of plasma were added to 600 µL of solvent.
- Samples were agitated for 30 minutes.
- 2 phases formed due to immiscibility of the solutions after agitation ceased.
- 300 µL of the top aqueous phase, representing plasma, was removed.
- 300 µL of plasma was diluted 10-fold with 25 mM AmBic.
- 60 mg of LRA was added to the samples.
- Samples were agitated at room temperature for 120 minutes.
- LRA was pelleted by centrifugation (14000 g for 5 minutes) at 120 minutes and the supernatant was removed.
- Supernatant was diluted 500-fold with ILMA and 100 µL was assayed for quantification of apoA1 concentration.

ApoA1 was completely depleted from the plasma, despite pre-treatment of plasma with the different solvents Table 3.6. The apoA1 concentrations were significantly higher in the plasma that was pre-treated with tetrachloroethylene, compared with the plasma control (112.38 mg/dL ± 5.99 vs. 95.53 ± 5.41; P=0.023 respectively). This difference may be due to the solvent's ability to solubilise the lipids, exposing more apoA1 epitopes, increasing antibody binding and thus the apoA1 signal. In contrast, the plasma incubated with chloroform had significantly lower apoA1 concentrations than the plasma control (48.00 mg/dL ± 9.84 vs. 95.53 ± 5.41; P=0.005 respectively). This was surprising as chloroform has been used in combination with methanol in 2:1 and has been proved to be an effective method of lipid extraction as described by Folch et al (Folch, Lees and Sloane Stanley, 1957).

Table 3.6 ApoA1 concentrations in plasma pre-treated with either tetrachloroethylene or chloroform prior to co-incubation with 20 mg/mL of LRA. Data values expressed as mean ± standard deviation, (n=3).

Sample	ApoA1 concentration (mg/dL)
Plasma control	95.53 ± 5.41
Pre-treatment of plasma with Tetrachloroethylene	112.38 ± 5.99
Pre-treatment of plasma with Chloroform	48.01 ± 9.84
Plasma co-incubated with LRA	0
Pre-treatment of plasma with Tetrachloroethylene prior to LRA co-incubation	0
Pre-treatment of plasma with Chloroform prior to LRA co-incubation	0

3.3.3.4 The influence of butanol and di-isopropyl ether (DIPE) on lipid binding to LRA

Triglycerides, phospholipids and fatty acids associate with proteins such as apolipoproteins, facilitating their transport. Various methods have been suggested for the isolation of lipids from the apolipoproteins, but have been complicated by protein precipitation and incomplete lipid removal, hampering any true interpretation of results (Scanu and Schiano, 1954). Cham et al suggested a novel method for removal of the lipids in a timely manner without significant protein precipitation. This method used a varying proportion of butanol and di-isopropyl ether (DIPE) creating a two phase solution when in contact with plasma; an organic phase containing the dissolved lipids, and an aqueous phase that contained the apolipoproteins in an unchanged state (Cham and Knowles, 1976). The mechanism by which the lipids bound to the affinity resin was explored by Cham et al., who hypothesised that, by pre-treating the plasma with butanol and DIPE, the lipids and in particular phospholipids would be removed, thereby limiting lipoprotein binding to the affinity resin, presumed to be via the phosphate group. Therefore, plasma was pre-treated with 2 different ratios of butanol and DIPE and then co-incubated with the affinity resin. ApoA1 levels were measured after a 2hour incubation time, detected by ELISA.

- Butanol (Sigma Aldrich) and DIPE (Sigma Aldrich) in ratios of 40:60 and 25:75 (v/v) were prepared.
- 600 µL of the organic mixtures were added to 300 µL of plasma.
- Samples were agitated for 1 hour at room temperature.
- Samples were centrifuged (14000 g for 5 minutes) and the top organic phase containing the dissolved lipid was removed.
- 700 µL of DIPE was added to the delipidated plasma to remove the trace of butanol, which can interfere with spectrophotometric analysis.
- Samples were agitated for 15 minutes at room temperature.
- Samples were then centrifuged (14000 g for 5 minutes) and the organic phase were removed.
- Further 700 µL of DIPE was added to the delipidated plasma.
- Samples were agitated for 15 minutes.
- Samples were centrifuged (14000 g for 5 minutes) and the organic phase were removed.
- 20 mg of LRA was pre-washed three times with 1 mL of 25 mM AmBic prior to plasma incubation.
- 100 µL of control plasma/ delipidated plasma was diluted 5-fold with 25 mM AmBic.
- Samples were incubated with 20 mg of pre-washed LRA.
- Samples were agitated for 2 hours at room temperature.
- LRA was pelleted by centrifugation (14000 g for 5 minutes) and the supernatant was removed.
- Samples were diluted 1000-fold with ILMA and 100 µL was assayed for determination of apoA1 concentrations.

The results are presented in Table 3.7. The delipidation of plasma using butanol and DIPE, prior to co-incubation with LRA, had no influence of apoA1 binding. The binding of apoA1 to the affinity resin was presumed via the interaction between the phosphate groups of phospholipids. This is via a different mechanism, as this method achieved complete depletion of phospholipids (100%) (Cham and Knowles, 1976).

Concentrations of apoA1 were significantly higher for the butanol and DIPE 40:60 and 25:75 preparations compared with control plasma (n=3, 142.76 mg/dL ± 12.35 vs. 40.06 ± 4.82; P=0.002 respectively) and (n=3, 145.52 mg/dL ± 4.99 vs. 40.06 ± 4.82; P<0.001 respectively). The solvent preparations yielded a more accurate representation of the true apoA1 concentration, consistent with the reference range of 94-178 mg/dL for males and 101-199 mg/dL for females. The solvents were likely to have disrupted the lipid-lipid interaction and lipid-protein interactions within the micelles, thereby exposing more apoA1 epitopes to the antibodies in the immunoassay.

The ability for the affinity resin to bind to apoA1, despite pre-treatment of plasma with solvents, suggests that this is a robust technique by which to explore the protein associated cargo of lipoproteins.

Table 3.7 The effect of different solvent pretreatments on the ApoA1 concentrations in delipidated plasma co-incubated with 40 mg/mL of LRA. Data values expressed as mean ± standard deviations, (n=3)

Sample	ApoA-1 concentration (mg/dL)
Plasma control	40.06 ± 4.81
Pre-treatment of plasma with 40:60 Butanol/DIPE	142.76 ± 12.35
Pre-treatment of plasma with 25:75 Butanol/DIPE	145.52 ± 4.99
Pre-treatment of plasma with 40:60 Butanol/DIPE co-incubated with LRA	1.86 ± 0.16
Pre-treatment of plasma with 25:75 Butanol/DIPE co-incubated with LRA	1.55 ± 0.35

3.3.4 Proteomic analysis of the lipid depleted plasma

3.3.4.1 Impact of prewashing LRA on protein binding

The impact of prewashing the affinity resin on protein binding was assessed. The LRA is a fine particulate resin, which may contain impurities, therefore it was hypothesised that pre-washing the resin prior to plasma co-incubation, would remove the impurities, which may interfere with MS analysis and reduce non-specific binding.

- 60 mg of LRA was prewashed three times with 1 mL of 25 mM AmBic.
- 300 µL of plasma was diluted 10-fold with 25 mM AmBic and added to 60 mg of prewashed and unwashed LRA.
- Samples were agitated for 2 hours at room temperature.
- LRA was pelleted by centrifugation (14000 g for 5 minutes) and the supernatant was removed.
- The LRA pellet was washed with 3 times with 1mL of 25 mM AmBic.
- Final 1 % sodium deoxycholate (SDC) (Sigma Aldrich) was added to linearize the proteins.
- Final concentration of 20 mM DTT (Sigma Aldrich) was added to each sample and incubated at 50 °C for 30 minutes.
- Final concentration of 20 mM IAA (Sigma Aldrich) was added to each sample and incubated in the dark at room temperature.
- Trypsin was added in a protein/enzyme ratio of 50:1 to allow digestion to occur on the LRA resin.
- Samples were incubated at overnight at 37 °C.
- The trypsin reaction was stopped by the addition of 0.1 % FA.

- LRA and SDC precipitates were pelleted by centrifugation (14000 g for 5 minutes) and the supernatant was removed for desalting using Empore C18 4 mm/1 ml catridges (Sigma Aldrich).

- SPE process involved initially priming the Empore C18 columns with 1 mL methanol and subsequently washing with 3 mL of 0.1 % FA prior to supernatant injection. The supernatant was injected and recirculated to increase peptide recovery. C18 columns were then washed 5 times with 1 mL of 0.1 % FA. Peptides were eluted with 1 mL of both 60 % and 80 % ACN in 0.1 % FA.

- Desalted samples were concentrated in a speed vac concentrator for 2 hours to evaporate the solvent and then lyophilised in a freezer drier overnight.
- Peptides were reconstituted in an equal volume of ADH (Waters Ltd.) and 0.1 % FA and analysed using LC-MS/MS (Thermo Scientific).

There was no significant difference in mean protein hits between the pre-washed and unwashed resin (mean 188 ± (SD) 1.14 vs. 186 ± 0; P=0.30 respectively) (Table 3.8). The hypothesis that prewashing the LRA would remove the impurities and therefore reduce non-specific binding was incorrect. Both protocols contained the complete array of apolipoproteins and the novel HDL associated proteins such as clusterin and PON-1 (Table 3.9). Pre-washing the affinity resin prior to plasma co-incubation was chosen for further experimentation on the premise that by removing the impurities from the affinity resin would improve MS analysis.

Table 3.8 Influence of pre-washing 20 mg/mL of affinity resin on protein hits, measured by the Thermo Q Exactive MS.

Sample	Protein hits
Unwashed LRA Sample 1	187
Unwashed LRA Sample 2	189
Pre-washed LRA Sample 1	186
Pre-washed LRA Sample 2	186

Table 3.9 Selected HDL-associated Proteins identified in the unwashed and pre-washed LRA samples.

Protein	Unwashed LRA	Pre-washed LRA
ApoA-1	+	+
ApoA-2	+	+
ApoA IV	+	+
ApoB100	+	+
ApoC-1	+	+
ApoC-II	+	+
ApoC-III	+	+
ApoD	+	+
ApoE	+	+
ApoF	+	+
ApoH	+	+
ApoL1	+	+
ApoM	+	+
Serum PON-1	+	+
Serum amyloid A-4	+	+
Apo(a)	-	-
Clusterin	+	+
Caeruloplasmin	+	+

3.3.4.2 The effect of detergents on the binding of proteins to LRA

The impact of detergents on the protein binding to the affinity resin was investigated. The non-ionic, non-denaturing detergents included NP40, Triton-X100 and Tween-20 (Sigma-Aldrich). Therefore, protein hits were measured in the plasma samples that were co-incubated with the affinity resin in the presence of the different detergents using the Thermo Q Exactive.

- 10 mg of LRA was pre-washed five times with 1 ml of PBS.
- 50 µL of plasma was diluted into 1ml of PBS or 0.1 % detergent in PBS
- 10 mg of LRA was added to each sample and incubated for 2 hours at room temperature.
- LRA samples were pelleted by centrifugation (14000 g for 5 minutes) and the supernatants were removed.

- LRA was washed 4 times with 1 mL of PBS or 0.1 % of the respective incubation detergent in PBS
- All samples were then washed 4 times with 1 mL PBS.
- The wash supernatant was removed and replaced with 500 µl of 1 % SDC and 10 mM Tributylphospine (TBP) which was used to linearize and reduce the proteins.
- Samples were incubated for 30 minutes at 50 °C.
- IAA was added to each sample at a final concentration of 50 mM and incubated in the dark at room temperature for 30 minutes.
- 50 µg of trypsin was added to each sample and incubated at 37 °C overnight.
- The trypsin reaction was stopped by the addition of concentrated trifluoroacetic acid (TFA) to attain a final concentration of 1 %.
- LRA and SDC precipitates were pelleted by centrifugation (14000 g for 5 minutes).
- The supernatant was subsequently desalted and lyophilised as described in 3.3.4.1.
- Peptides were reconstituted in an equal volume of ADH and 0.1% FA and analysed using LC-MS/MS.

The protein hits for the different sample protocols are presented in Table 3.10. There was no significant difference in protein hits between the different protocols (P=0.392). Qualitatively, incubation with NP40 and Triton contained the full complement of the HDL associated proteins, with the additional identification of apo(a), which is adversely linked with CAD (Table 3.11). Both NP40 and Triton samples were depleted of ceruloplasmin, suggesting that the detergents possibly reduced non-specific binding to the resin.

The Venn diagram in Figure 3.2, demonstrates the homology between the different sample preparations, with 87 proteins universally identified in the control, NP40 and Triton samples. The control sample identified 21 unique proteins compared to the 3 proteins identified for NP40 and the 5 for Triton. The use of such detergents posed a significant challenge to MS throughput, as increased washing times were required between sample runs for column reconditioning. Such detergents were therefore avoided in subsequent experiments.

Table 3.10 Protein hits for plasma co-incubated with 10 mg/mL of LRA in the presence of AmBic NP40, tween and triton.

Sample	Different proteins identified
LRA Control	126
LRA NP40	104
LRA Tween	119
LRA Triton	106

Table 3.11 Qualitative assessment of the HDL-associated proteins identified in plasma co-incubated with 10 mg/mL of LRA in the presence of AmBic, NP40, tween and triton.

Protein	Control	NP40	Tween	Triton
ApoA-1	+	+	+	+
ApoA-2	+	+	+	+
ApoB100	+	+	+	+
ApoC-1	+	+	+	+
ApoC-III	+	+	+	+
ApoD	+	+*	+	+
ApoE	+	+	+	+
ApoF	+	+	+	-
ApoH	+	+	+	+
ApoL1	+	+	+	+
ApoM	+	-	+	+#
Serum PON-1	+	+	+	+
Serum amyloid A-4	++	+	+	+
Apo(a)	-	+	-	+
Clusterin	+	+	+	+
Caeruloplasmin	+	-	+	-

+ denotes present; - denotes absent; * denotes protein probability 50-79%; + denotes protein probability 80-94%; # denotes protein probability 20-49%

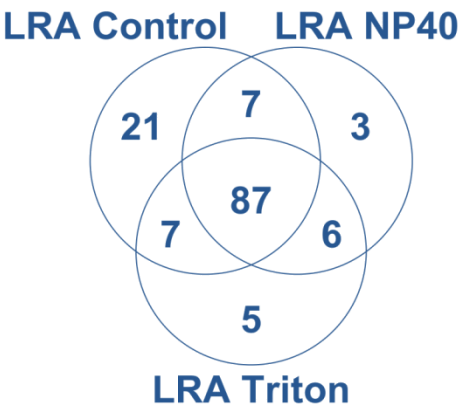


Figure 3.2 Venn diagram showing the common proteins identified between the plasma samples co-incubated with LRA and the AmBic, NP40 and triton treated media

3.3.4.3 The impact of washing LRA on protein binding

An alternative method for limiting non-specific binding to the affinity resin was investigated, by assessing the impact of washing the affinity resin after plasma incubation. It was hypothesised that washing the affinity resin after plasma incubation would minimise non-specific protein binding, thereby enhancing the coverage of the low abundant proteins detected by MS.

- 10 mg of LRA was prewashed 5 times with 1 mL of 50 mM AmBic.
- 50 µL of plasma was diluted 5-fold with 50 mM AmBic.
- 10 mg of prewashed LRA was added to the diluted plasma sample.
- Samples were agitated for 2 hours at room temperature.
- LRA was pelleted by centrifugation (14000 g for 5 minutes).
- The LRA was washed 8 times with 1 mL of 50 mM AmBic and the washes were collected.
- Samples were reduced with DTT, alkylated and digested as described previously in 3.3.4.1.
- Samples were desalted, and lyophilised as described previously in 3.3.4.1

For analytical simplicity, the washes were combined into 2 collections. The initial 1st wash was discarded, as it was likely to contain a large amount of albumin and potentially hamper MS analysis. The 2nd and 3rd washes were combined into a single sample, whilst the remaining 4th-8th washes were combined into a further sample. The protein concentrations and protein hits for the different samples are presented in Figure 3.3 & Table 3.12 respectively. It was evident that the process of washing the affinity resin in a neutral buffer removed 19 uniquely identified proteins, Figure 3.4 and Table 3.13. The remaining washes failed to remove any further proteins from the resin. It was therefore argued that washing the affinity resin a minimum of three times was sufficient to remove the weakly adsorbed proteins from its surface.

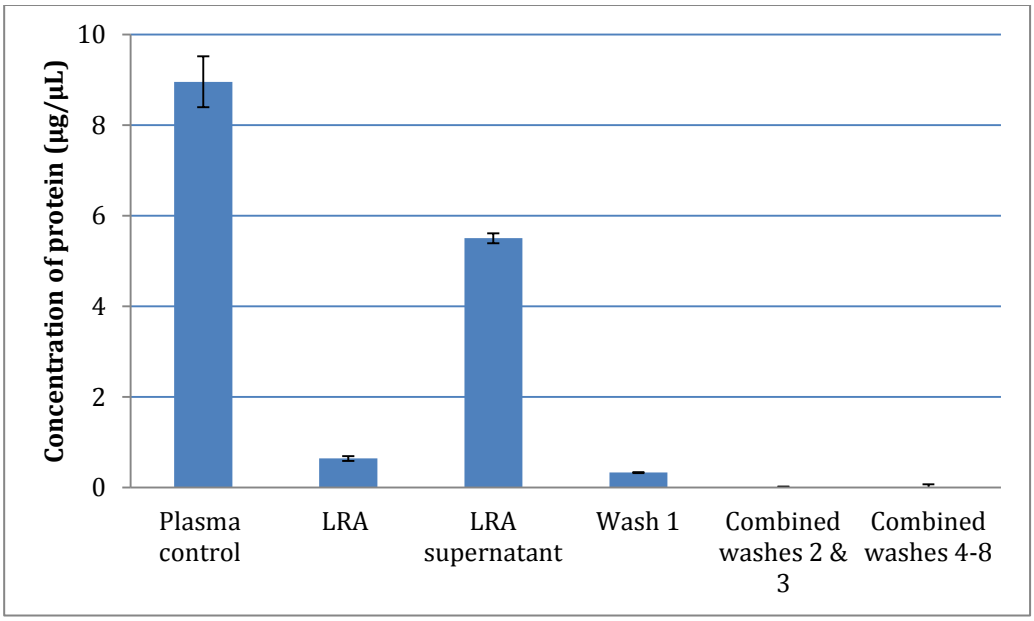


Figure 3.3 Bar chart showing the protein concentrations in the plasma co-incubated with 40 mg/mL of LRA samples, determined using the BCA protein assay (n=3).

Table 3.12 Proteins identified in the plasma samples co-incubated with 40 mg/mL of LRA in the presence of AmBic and the post plasma incubation washes.

Sample	Protein Hits
LRA **Replicate 1** **Replicate 2** **Replicate 3**	 111 105 112
LRA 2nd/3rd Washes	76
LRA 4th-8th Washes	24

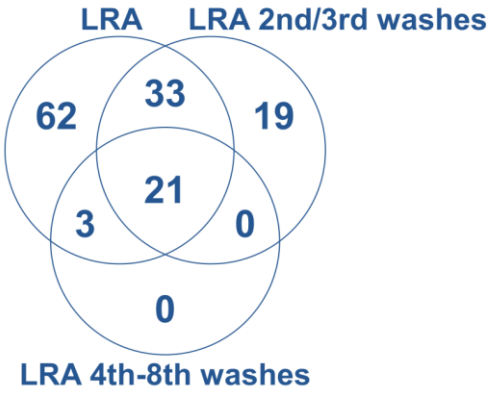


Figure 3.4 Venn diagram showing the number of different proteins identified for the plasma samples co-incubated with LRA in the presence of AmBic and the post plasma incubation washes, using 1D LC-MS/MS.

Table 3.13 Proteins only found in the 2nd/3rd LRA wash using 1D LC-MS/MS.

	Protein Name	Accession Number
1	Alpha-1B-glycoprotein	P04217
2	Zinc-alpha-2-glycoprotein	P25311
3	Leucine-rich alpha-2-glycoprotein	P02750
4	Alpha-1-acid glycoprotein	P02763
5	CST complex subunit STN1	Q9H668
6	Spectrin alpha chain, erythrocytic 1	P02549
7	LINE-1 type transposase domain-containing protein 1	Q5T7N2
8	Kinesin-like protein KIF16B	Q96L93
9	Keratin, type II cytoskeletal 1	P04264
10	Keratin, type I cytoskeletal 10	P13645
11	Ig kappa chain V-I region AG	P01593
12	Biotinidase	P43251
13	Myosin-10	P35580
14	Centrosomal protein of 120 kDa	Q8N960
15	Ankyrin repeat domain-containing protein 36A	A6QL64
16	Hemoglobin subunit delta	P02042
17	Ig kappa chain V-II region RPMI 6410	P06310

18	Ig heavy chain V-III region WEA	P01763
19	Ig kappa chain V-I region WEA	P01610

3.3.4.4 **Increasing the coverage of low abundant proteins using MARS**

LRA is a resin capable of isolating the lipoproteins and their associated cargo. Exposing the lipid depleted plasma to a further method of separation may reduce the complexity and the dynamic range of the sample, leading to greater coverage of low abundant proteins. In this experiment, the lipid depleted supernatant was fractionated into high and low abundant fractions, using the MARS column (Agilent Technologies (Stockport, UK), which specifically removes the top 14 most abundant proteins.

- 50 µL of plasma was diluted 5-fold with 50 mM AmBic.
- 10 mg of prewashed LRA was added to the diluted plasma sample.
- Samples were agitated for 2 hours at room temperature.
- LRA was pelleted by centrifugation and the supernatant was removed.
- Prior to injection into the MARS column (Agilent Technologies), 70 µL of plasma control was diluted 5 times with buffer A (Agilent Technologies).
- LRA supernatant and plasma samples were placed in 0.22 µm spin filters and centrifuged for 1 minute to remove particulate matter.
- HPLC was calibrated prior to sample run.
- 200 µL of supernatant/plasma samples were injected into the MARS column.
- High and low abundance protein fractions were collected.
- Fractions were placed in Amicon spin filter tubes (Millipore) and spun for 15 minutes at 2500 G to remove proteins below 5 KDa.

- Using the Amicon spin filters, samples were co-incubated with 50 mM AmBic and spun at 2500 G for 15 minutes and repeated three times in a process known as buffer exchange.
- Samples were reduced, alkylated, digested as previously described in 3.3.4.1.
- Samples were desalted, concentrated and lyophilised as previously described in 3.3.4.1.
- Peptides were reconstituted in an equal volume of ADH (Waters Ltd.) and 0.1 % FA and analysed using LC-MS/MS (Thermo Scientific).

Unexpectedly, the protein hits for the low abundant fraction of the LRA supernatant were significantly lower, than for the low abundant fraction of the untreated plasma (n=3, mean 59.7 ± (SD) 5.5 vs. 112 ± 1.0; P=0.03) respectively. Similarly, the mean protein hits for the high abundant fraction of the LRA supernatant were also significantly lower, than the high abundant fraction of the untreated plasma (n=3, mean 50.7 ± (SD) 1.5 vs. 62.7 ± 1.2; P<0.001 respectively). Although, the proteins numbers were significantly lower for both fractions of the LRA supernatant, the greatest discrepancy occurred between the low abundant fraction of the LRA supernatant and the low abundant fraction of the control plasma. This potentially suggests that the low abundant proteins may bind preferentially to LRA, over the high abundant proteins. LRA may therefore act as a lipoproteomic enrichment resin.

The hypothesis that the depletion of lipids and lipoproteins from plasma using the affinity resin would effectively reduce the dynamic range of the supernatant and improve coverage of the low abundant proteins was incorrect. From the Venn diagram in Figure 3.5 is evident that 49 proteins were homologous between the affinity resin, low abundant fraction of the LRA supernatant and the low abundant fraction of the plasma control. The affinity resin identified 15 unique proteins compared with 12 for the low abundant fraction of the LRA supernatant.

Table 3.14, Table 3.15 and Table 3.16 display the unique proteins for the different samples. The diversity in the identified proteins between the samples is reflective of different preparative methods. Each method exploits different physicochemical attributes of the lipoproteins and their associated protein cargo.

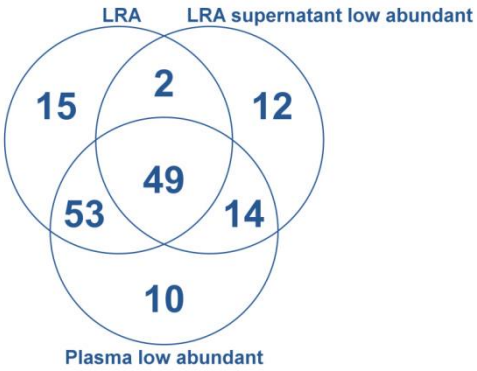


Figure 3.5 Venn diagram showing the number of different proteins identified for the LRA resin, low abundant fraction of the LRA supernatant and the low abundant fraction of plasma samples

Table 3.14 Proteins only found in the LRA samples

	Protein Name	Accession Number
1	Ig lambda chain V-III region SH	P01714
2	Transmembrane protein 198	Q66K66
3	ATP-binding cassette sub-family A member 12	Q86UK0
4	Ig lambda chain V-III region LOI	P80748
5	Ig kappa chain V-III region VH (Fragment)	P04434
6	Ig heavy chain V-III region BUT	P01767
7	Coiled-coil domain-containing protein 168	Q8NDH2
8	Ig heavy chain V-III region TIL	P01765
9	Ig kappa chain V-III region CLL	P04207
10	Ig heavy chain V-III region GAL	P01781
11	Fibulin-1	P23142
12	Coiled-coil domain-containing protein 73	Q6ZRK6
13	Ig kappa chain V-IV region Len	P01625
14	Complement C1q subcomponent subunit B	P02746
15	Extracellular matrix protein 1	Q16610

Table 3.15 Proteins only found in the low abundant fraction of the LRA supernatant

	Protein Name	Accession Number
1	Lysine-specific demethylase 5A	P29375
2	Dopamine beta-hydroxylase	P09172
3	Complement factor H-related protein 4	Q92496
4	Platelet glycoprotein Ib alpha chain	P07359
5	Intercellular adhesion molecule 1	P05362
6	Fibrous sheath-interacting protein 2	Q5CZC0
7	Structural maintenance of chromosomes flexible hinge domain-containing protein 1	A6NHR9
8	CD166 antigen	Q13740
9	Neural cell adhesion molecule L1-like protein	O00533
10	Centrosomal protein of 120 kDa	Q8N960
11	Ankyrin repeat domain-containing protein 36A	A6QL64
12	G protein-coupled receptor kinase 5	P34947

Table 3.16 Proteins only found in the low abundant fraction of Plasma

	Protein Name	Accession Number
1	CST complex subunit STN1	Q9H668
2	Heparin cofactor 2	P05546
3	Plasma kallikrein	P03952
4	Complement C2	P06681
5	Insulin-like growth factor-binding protein complex acid labile subunit	P35858
6	Corticosteroid-binding globulin	P08185
7	GXP 3	P22352
8	Serum amyloid P-component	P02743
9	Carboxypeptidase B2	Q96IY4
10	Biotinidase	P43251

3.3.4.5 **The extraction of the LRA bound proteins**

The selective removal of the hydrophobic proteins adsorbed onto the affinity resin was assessed using detergents such as sodium dodecyl sulphate (SDS), SDC and the cosolvent trifluoroethanol (TFE) in an attempt to maximise protein coverage.

- 400 µL of plasma was diluted 10-fold with 50 mM AmBic and added to the 160 mg of prewashed LRA.
- Samples were agitated for 2 hours at room temperature.

- LRA was pelleted by centrifugation (14000 g for 5 minutes) and the supernatant was removed.
- LRA resin was washed 3 times with 4 mL of 50 mM AmBic to reduce non-specific binding.
- The LRA sample was then split equally into four tubes and the wash solution was replaced with 50 mM AmBic, 1 % SDS (final concentration) (Sigma Aldrich), 1 % SDC (final concentration) and 50 % TFE (final concentration) (Sigma Aldrich).
- Samples were reduced and alkylated as previously described in 3.3.4.1.
- SDS and TFE samples were diluted with 50 mM AmBic achieving concentrations below 0.07 % and 10 % respectively, compatible with trypsin digestion.
- 200 mg of trypsin was added and incubated overnight at 37 °C.
- The trypsin reaction was stopped by the addition of concentrated TFA to attain a 1 % final concentration.
- As SDS interferes with MS analysis, an equal volume of 0.5 M potassium chloride (KCl) was added to each sample to enhance the precipitation of SDS.
- Samples were subsequently desalted and lyophilised as previously described in 3.3.4.1.
- Peptides were reconstituted in an equal volume of ADH (Waters Ltd.) and 0.1 % FA and analysed using LC-MS/MS (Thermo Scientific).

The proteins hits for the different methods are displayed in Table 3.17. From the results, it is evident that the SDS treatment resulted in a significant loss of proteins compared to the other methods. The low protein hits observed in the SDS sample was likely to be due to ion suppression from persisting SDS particles. The use of a 2 step clean-up process has been previously documented, to achieve impressive removal of SDS, without a significant impact on protein hits. The precipitation of SDS salts with KCl was reported to completely remove the SDS from the sample whilst achieving 96 % peptide recovery (Zhou *et al*, 2012). In addition, the subsequent desalting step has been reported to achieve 80 % peptide recovery, which was far superior to that observed for other methods of SDS removal such as

acetone precipitation (Zhou *et al*, 2012). The persistence of SDS in the sample may have been due to formation of salt bridges between the sulphate groups on SDS and the amino acids on hydrophobic proteins making them resistant to such clean-up processes (Zhou *et al*, 2012). Zhou et al. also commented that the signal from hydrophobic proteins was significantly reduced, which was likely to be a result of protein precipitation from the KCl (Zhou *et al*, 2012). The vast majority of the lipoproteins contain hydrophobic protein cargos, which potentially could be obscured when experimenting with SDS, for this reason further experimentation with SDS was avoided.

By contrast, SDC is compatible with MS analysis and did not suppress ionisation. The mean protein hits for the SDC samples were comparable with the AmBic control samples (n=3, mean 155.3 ± (SD) 2.2 vs. 139.0 ± 19.2; P=0.144 respectively). There was also equivalence in protein hits between the TFE treated samples and the control samples (n=3, 159.3 ± 1.7 vs. 139.0 ± 19.2; P=0.080 respectively). Between the treated samples, TFE yielded significantly higher proteins hits than the SDC samples (n=3, 159.3 ± 1.7 vs. 155.3 ± 2.2; P=0.029 respectively), suggesting that the co-solvent had greater selective ability in removing the hydrophobic proteins from the resin.

Qualitatively, all three methodologies yielded the entire complement of apolipoproteins and the common HDL associated proteins Table 3.18. One hundred and sixty one proteins were common to all three samples, with 11, 4 and 8 unique proteins identified in the control, SDC treated and TFE treated samples respectively (Figure 3.6, Table 3.19-3.21). Surprisingly, the TFE treated samples failed to identify more novel proteins over the other methods, considering that its solvation properties are ideally suited for the hydrophobic proteins.

Each protocol had a set of unique proteins which must be offset against the practical implication of processing that sample. The use of SDS, although a widely used solubilising detergent, caused significant ion suppression in this experiment, despite the clean-up processes. The use of TFE posed a challenge, as the initial concentration of 50%, required for extraction of the hydrophobic proteins from the resin, had to be diluted to below 0.07 % to prevent trypsin denaturation. Such

dilutions led to creation of large sample volumes, which added considerable time to the desalting process.

The use of SDC posed no challenge to sample throughput or to MS analysis. Quantitative and qualitative analysis of the proteins identified in the SDC treated samples were comparable with the control samples. Therefore, subsequent experimentation with SDC was undertaken.

Table 3.17 Number of proteins identified for the plasma samples co-incubated with 40 mg/mL of LRA in the presence of AmBic, SDS, SDC and TFE.

Protocol	Peptide loaded (µg)	Different proteins identified
LRA AmBic Control – 60% elution		
Replicate 1 Replicate 2 Replicate 3	1ug	133 118 141
LRA AmBic Control – 80% elution		
Replicate 1		164
LRA 1% SDS – 60% elution		
Replicate 1		7
LRA 1% SDC –60% elution		
Replicate 1 Replicate 2 Replicate 3	1ug	158 154 156
LRA 1% SDC –80% elution		
Replicate 1		153
LRA 50% TFE - 60% elution		
Replicate 1 Replicate 2 Replicate 3	1ug	157 161 160
LRA 50% TFE – 80% elution		
Replicate 1		159

Table 3.18 HDL-associated proteins identified in the plasma samples co-incubated with LRA in the presence of AmBic, SDS, SDC and TFE.

Protein	Control	1% SDS	1% SDC	50% TFE
ApoA-1	+	+*	+	+
ApoA-2	+	-	+	+
ApoA-4	+	-	+	+
ApoB100	+	-	+	+
ApoC-1	+	-	+	+
ApoC-2	+	-	+	+
ApoC-III	+	-	+	+
ApoD	+#	-	+°	+
ApoE	+	-	+	+
ApoH	+°	-	+	+
ApoL1	+	-	+	+
ApoM	+	-	+	+
Serum PON-1	+	-	+	+
Serum PON-3	-	-	-	-
Serum amyloid A-1	+	-	+	+
Serum amyloid A-4	+	-	+°	+
Serum amyloid P	+	-	+	+
Apo(a)	-	-	-	-
Clusterin	+	-	+	+
Caeruloplasmin	+	-	+	+

+ denotes present; - denotes absent; * denotes protein probability 50-79%; + denotes protein probability 80-94%; # denotes protein probability 20-49%; √° denotes present in the 80% elution.

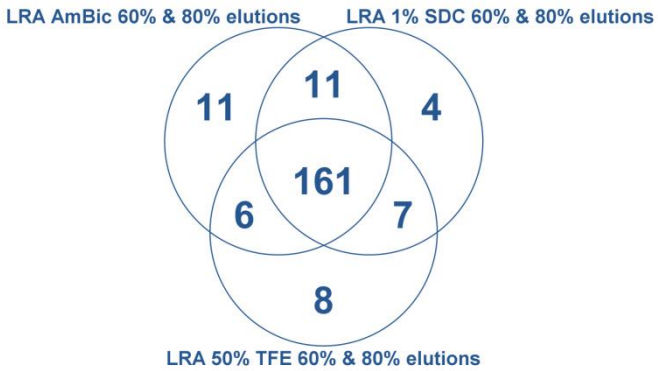


Figure 3.6 Venn diagram showing the number of different proteins identified for the plasma samples co-incubated with LRA in the presence of AmBic, 1% SDC and 50% TFE, using 1D LC-MS/MS.

Table 3.19 Proteins only found in the AmBic extracted samples. Proteins identified with a 1% FDR criteria.

		Accession Number
1	RAS guanyl-releasing protein 1	O95267
2	Sperm-specific antigen 2	P28290
3	Kinesin-like protein KIF26B	Q2KJY2
4	Collectin-11	Q9BWP8
5	Probable E3 ubiquitin-protein ligase HERC3	Q15034
6	Heterochromatin protein 1-binding protein 3	Q5SSJ5
7	Piwi-like protein 2	Q8TC59
8	Insulin-like growth factor-binding protein 3	P17936
9	GTPase IMAP family member 8	Q8ND71
10	78 kDa glucose-regulated protein	P11021
11	Kinesin-like protein KIF14	Q15058

Table 3.20 Proteins only found in the 1% SDC extracted samples. Proteins identified with a 1% FDR criteria.

	Protein Name	Accession Number
1	Intraflagellar transport protein 122 homolog	Q9HBG6
2	Kinetochore protein Nuf2	Q9BZD4
3	Ryanodine receptor 3	Q15413
4	Hypoxia up-regulated protein 1	Q9Y4L1

Table 3.21 Proteins only found in the 50% TFE extracted samples Proteins identified with a 1% FDR criteria.

	Protein Name	Accession Number
1	Complement C1q subcomponent subunit C	P02747
2	Ig heavy chain V-II region ARH-77	P06331
3	Vacuolar protein sorting-associated protein 13D	Q5THJ4
4	Properdin	P27918
5	Pleckstrin homology domain-containing family H member 1	Q9ULM0
6	Hepatocyte growth factor-like protein	P26927
7	EGF-containing fibulin-like extracellular matrix protein 1	Q12805
8	Galactose-1-phosphate uridylyltransferase	P07902

3.3.4.6 **The effect of temperature on protein hits**

The effect of incubating the plasma/affinity resin complex at different temperatures on protein coverage, prior to the digestion, was investigated. It was hypothesised that thermal exposure to the AmBic control and SDC treated samples, would denature the bound proteins and expose the previously obscured epiptopes for tryspin digestion, enhancing protein coverage. The impact of overnight incubation was also investigated, to assess its impact on protein coverage.

- 50 µL of plasma was diluted 5-fold with 50 mM AmBic and added to the 10 mg of prewashed LRA.
- Samples were agitated for either 2 hours or overnight at room temperature.
- LRA was pelleted by centrifugation (14000 g for 5 minutes) and the supernatant was removed.
- LRA resin was washed 3 times with 1 mL of 50 mM AmBic to reduce non-specific binding.
- The wash solution was replaced with 50 mM AmBic or 1 % SDS.
- Samples were then incubated at 60°C, 90°C and 120°C for 10 minutes.
- Samples were reduced, alkylated and digested as previously described in 3.3.4.1.
- Samples were subsequently desalted and lyophilised as previously described in 3.3.4.1.
- Peptides were reconstituted in an equal volume of ADH (Waters Ltd.) and 0.1% FA and analysed using LC-MS/MS (Thermo Scientific).

The results are presented in Table 3.22 & Figure 3.7. The mean number protein hits for control samples incubated at 60 °C and 90 °C were significantly higher, than the SDC treated samples at 60 ˚C and 120 °C. A maximum temperature of 90 °C for the control samples was chosen, to avoid potential protein precipitation. A higher maximum temperature of 120 °C was chosen for the SDC samples, due to its solubilising properties. There was no significant difference in protein hits between

the control samples incubated at 60 °C and 90 °C (n=3, 163.67 ± 2.08 vs. 160.00 ± 2.89; P=0.13 respectively). The mean protein hits for the control samples incubated at 60 °C were significantly higher than the SDC treated samples incubated at 60 °C (n=3, 163. 67 ± 2.08 vs. 153.00 ± 3; P=0.005). Similarly, the control sample incubated 60 °C gave significantly higher protein hits than the SDC treated samples incubated at 120 °C (n=3, 163. 67 ± 2.08 vs. 152.00 ± 2.65; P=0.004). The lower hits in the SDC treated samples were likely to represent loss of proteins at the time of SDC precipitation. The overnight incubation conferred no significant improvement in proteins hits for neither of the control samples incubated at 60 °C or 90 °C. Based on these findings, the control sample of AmBic incubated for 2 hours with plasma with subsequent 10minute incubation at 60 °C prior to reduction, alkylation and digestion was chosen as the optimal method for proceeding with the analysis of the clinical samples.

Table 3.22 Number of proteins identified for the plasma samples co-incubated with 40 mg/mL of LRA in the presence of AmBic and SDC with time and temperature differentials.

Sample	Peptide loaded (µg)	Different proteins identified
LRA AmBic 60 °C, Overnight incubation		
Replicate 1	1 ug	161
Replicate 2	1 µg	165
Replicate 3	1 ug	163
LRA AmBic 60 °C. 2 hour incubation		
Replicate 1	1 ug	166
Replicate 2	1 µg	162
Replicate 3	1 ug	163
LRA AmBic 90 °C, Overnight incubation		
Replicate 1	1 ug	161
Replicate 2	1 µg	156
Replicate 3	1 ug	162
LRA AmBic 90 °C, 2 hour incubation		
Replicate 1	1 ug	162
Replicate 2	1 µg	157
Replicate 3	1 ug	161
LRA SDC 60 °C, Overnight incubation		
Replicate 1	1 ug	164
Replicate 2	1 µg	159
Replicate 3	1 ug	159
LRA SDC 60 °C, 2 hour incubation		
Replicate 1	1 ug	156
Replicate 2	1 µg	153
Replicate 3	1 ug	150

LRA SDC 120 °C, Overnight incubation		
Replicate 1	1 ug	157
Replicate 2	1 µg	154
Replicate 3	1 ug	155
LRA SDC 120 °C , 2 hour incubation		
Replicate 1	1 ug	150
Replicate 2	1 ug	155
Replicate 3	1 ug	151

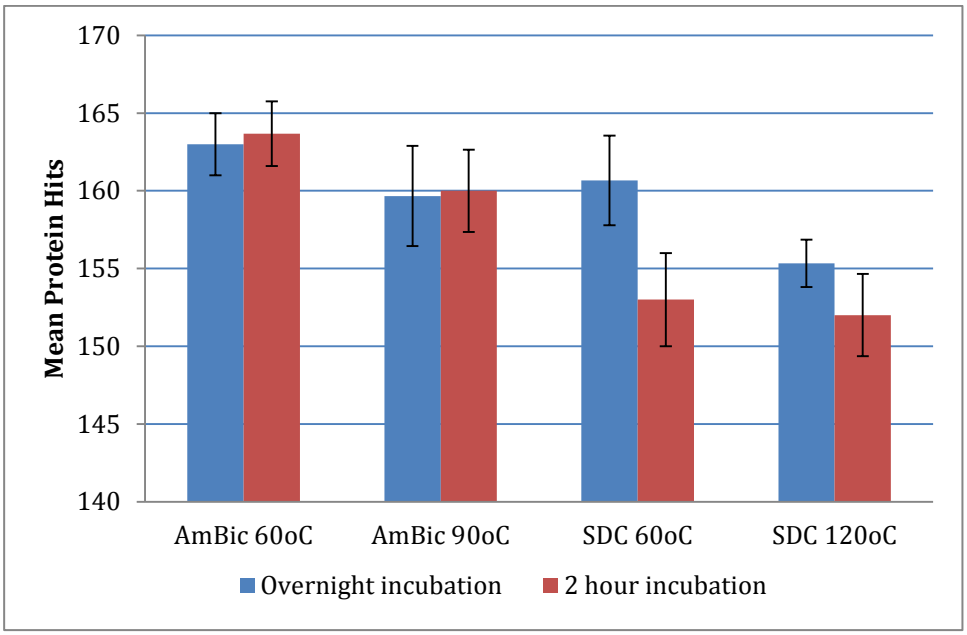


Figure 3.7 Bar chart showing the protein hits for the plasma samples co-incubated with LRA in the presence of AmBic and SDC with time and temperature differentials. Data expressed as mean ± standard deviation.

3.3.4.7 **Assessment of process of desalting**

The complexity of plasma poses a real challenge for the identification of the low abundant proteins and ultimately biomarker discovery. The processing of plasma via the depletion of the high abundant proteins or conversely the enrichment of the low abundant proteins facilitates biomarker discovery. Sample processing can add to the complexity of MS data analysis. Changes to the initial sample may distort the final results, as protein identification and even expression may not be truly reflective of the actual sample, but a consequence of the processing steps. For this reason, the complexity of sample preparation was streamlined, by evaluating the necessity of SPE. This process serves to remove the salts, which may interfere with MS analysis, at the expense of potential protein loss or irreproducibility.

Previous experimentation with detergents required desalting, however as sample preparation has evolved to leave out detergents, the need for SPE was evaluated.

- 50 µL of plasma was diluted 5-fold with 50 mM AmBic and added to 10 mg of prewashed LRA.
- Samples were agitated for either 2 hours or overnight at room temperature.
- LRA was pelleted by centrifugation (14000 g for 5 minutes) and the supernatant was removed.
- LRA resin was washed 3 times with 1 mL of 50 mM AmBic to reduce non-specific binding.
- Samples were then incubated at 60 °C for 10 minutes.
- Samples were reduced, alkylated and digested as previously described in 3.3.4.1.
- Half of the samples underwent SPE using Empore C18 4 mm/1 mL catridges (Sigma Aldrich), as previously described in 3.3.4.1 and the remainder moved onto the next stage.
- Sample were concentrated and lyophilised as previously described in 3.3.4.1.
- Peptides were reconstituted in an equal volume of ADH (Waters Ltd.) and 0.1% FA and analysed using LC-MS/MS (Thermo Scientific).

The protein hits for the samples are presented in Figure 3.8. There was no significant difference in mean protein hits between the solid phase extracted samples and the non-solid phase extracted samples (132.75 ± 5.00 vs. 136.00 ± 3.07; P=0.310). The coefficient of variation (CV) in the protein hits for both protocols, were below the acceptable 15 % cut off as depicted in Figure 3.9. Furthermore, the number of peptides identified for randomly selected proteins were comparable between the two protocols, Figure 3.10. The dynamic ranges of protein concentrations conferred by both protocols were similar (Figure 3.11). In view of this, it was decided that the process of desalting was not necessary for the sample preparation and was therefore omitted from the experimental design.

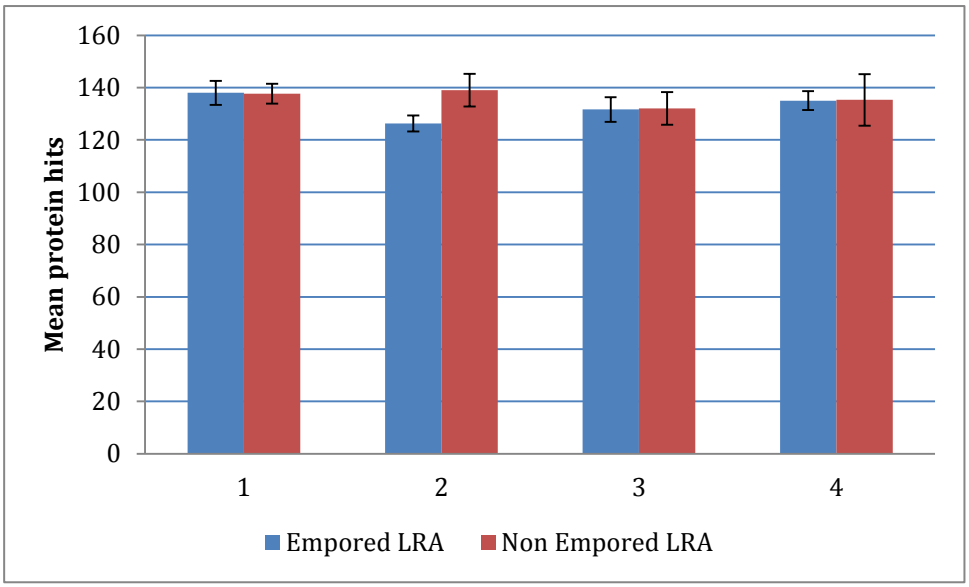


Figure 3.8 Bar chart showing the protein hits for the 4 solid phase extracted (C18 Empored) LRA (40 mg/mL) samples and the 4 non-solid phase extracted LRA (40 mg/mL) samples. Data expressed as mean ± standard deviation, (n=3).

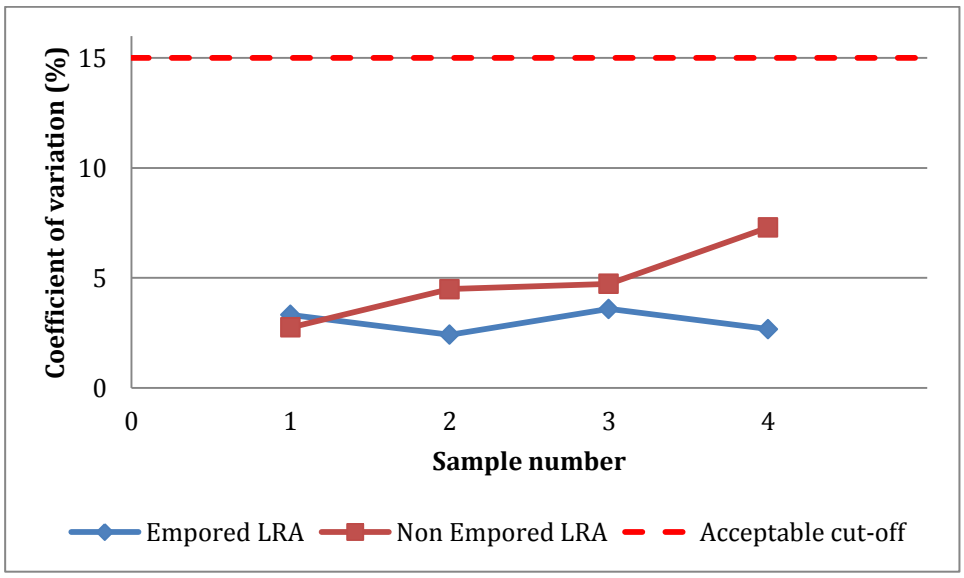


Figure 3.9 Coefficient of variation in the protein hits for the solid phase extracted samples and the non-solid phase extracted samples

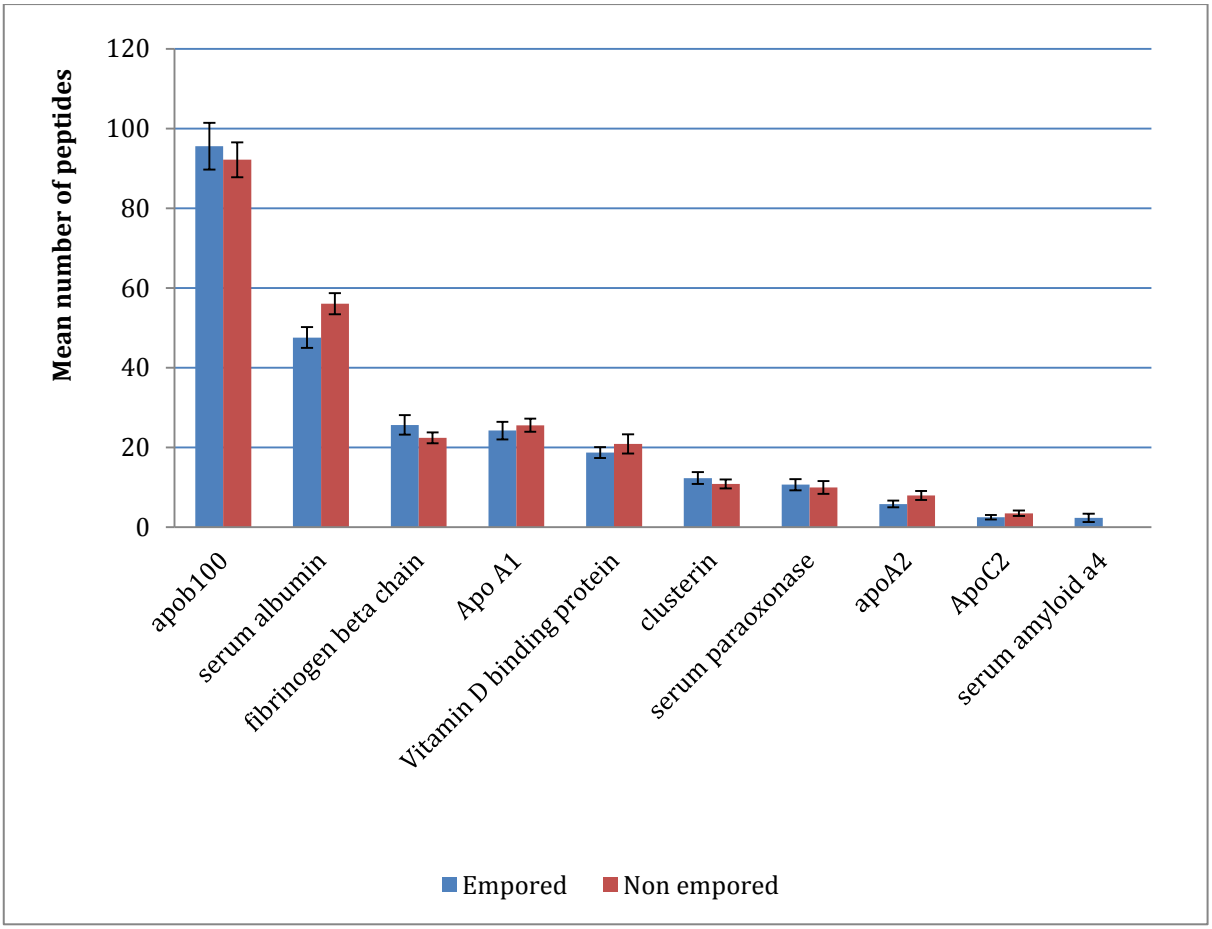


Figure 3.10 Bar charts showing the number of peptides identified for randomly selected protein for the solid phase extracted samples and the non-solid phase extracted samples. Data are expressed as mean ± standard deviation.

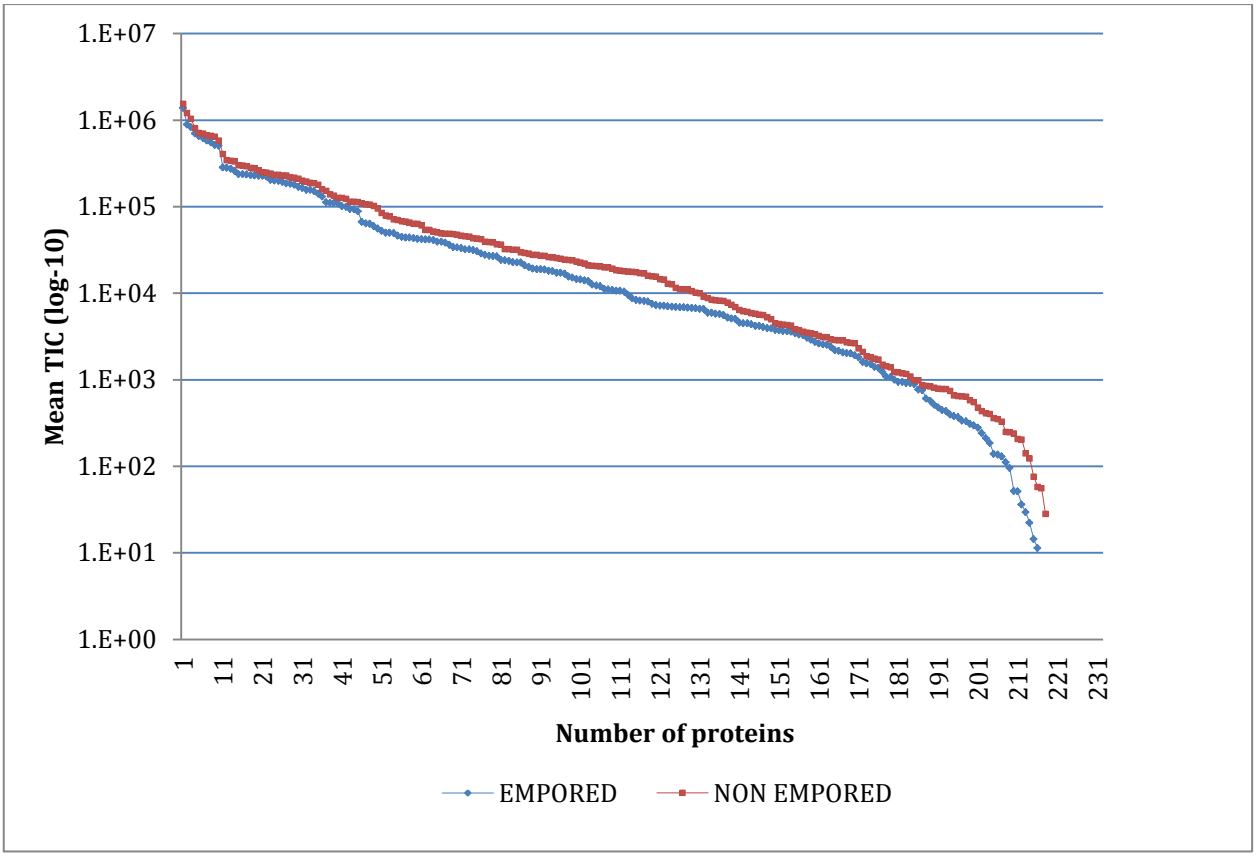


Figure 3.11 Scatter graph showing the dynamic range for the proteins identified in the solid phase (Empored) extracted and non-solid phase extracted samples.

3.4 Summary of Findings

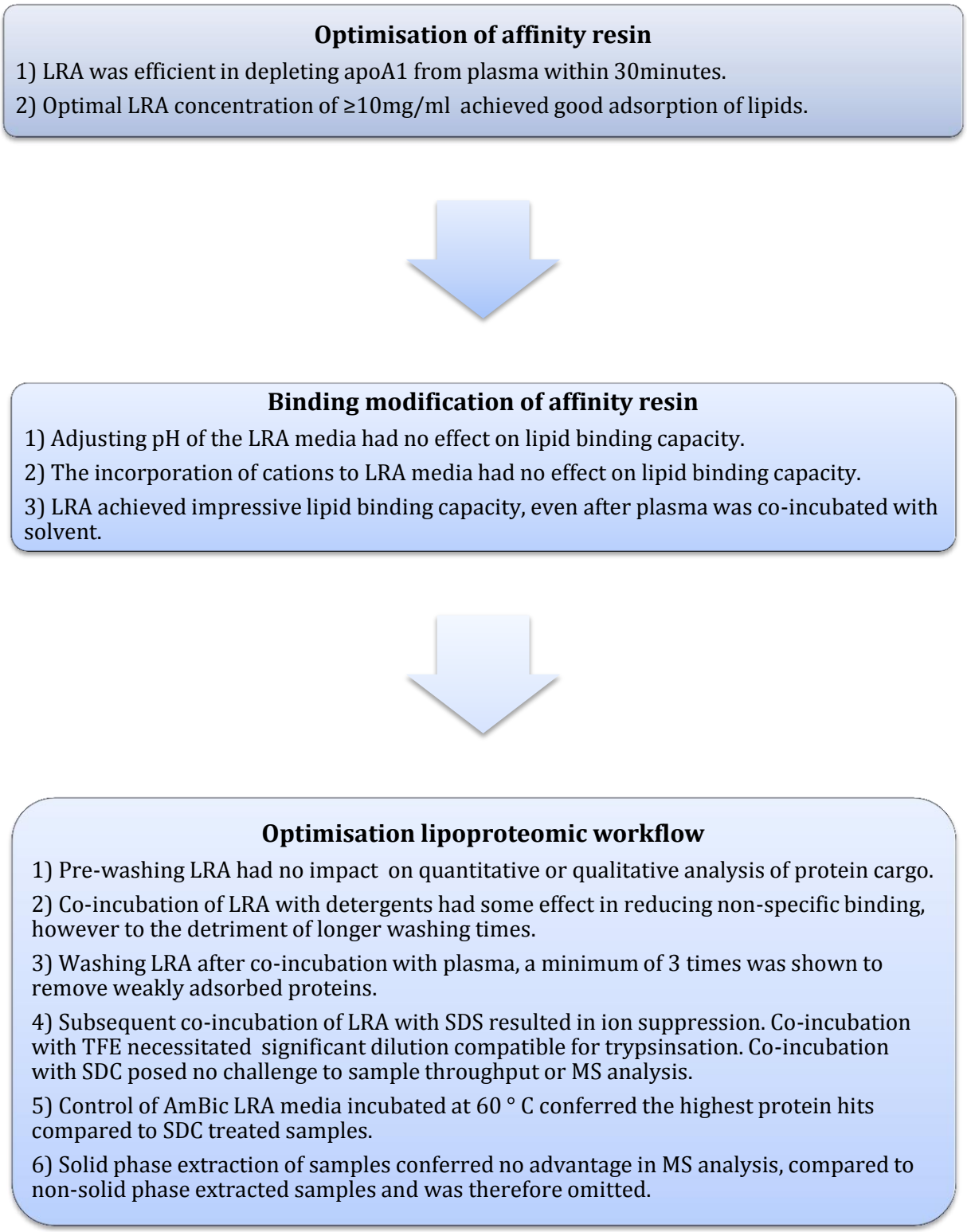


Figure 3.12 Summary workflow of the experimental process

Chapter 4

4 The influence of statin therapy on lipoprotein associated proteins in patients with hypercholesterolemia

4.1 Introduction

The epidemiological evidence directly linking LDL-cholesterol (LDL-C) and total cholesterol to CAD is well founded. The introduction of statins, which inhibit cholesterol biosynthesis and the downstream cholesterol products, has had a huge impact in improving the survival of patients post myocardial infarction. Interestingly, it was first noted in the West of Scotland Coronary Prevention Study (WOSCOPS) that the survival benefits conferred by statins, could not solely be attributable to the reductions in LDL-C alone (Shepherd *et al*, 1995). In this study patients with moderate hypercholesterolemia were randomly assigned to either pravastatin 40 mg or placebo for primary prevention. The treated group, as expected had reductions in total cholesterol and LDL by 20 % and 26 % respectively. This translated to reductions in both coronary events and all-cause mortality by 31 % and 32 % respectively, compared to placebo groups. Unexpectedly, the Framingham risk score underestimated the risk reduction, based on the LDL reduction in the treatment group (24 %), but accurately estimated the risk reduction for the placebo group. This discrepancy suggested that statins were mediating incremental health benefits, beyond their lipid lowering capability, which has led to the appreciation that statins have lipid independent or pleiotropic effects ('Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS)', 1998).

The cholesterol biosynthetic pathway is complex, such that the inhibition of the HMGCoA reductase enzyme disturbs the downstream products and pathways. HMGCoA reductase is responsible for the conversion of HMGCoA to mevalonate which is a precursor for the prenylation of various proteins contributing to cellular

signalling (Schafer and Rine, 1992). It is this disturbance that is believed to be responsible for the myriad of pleiotropic effects that statins exert.

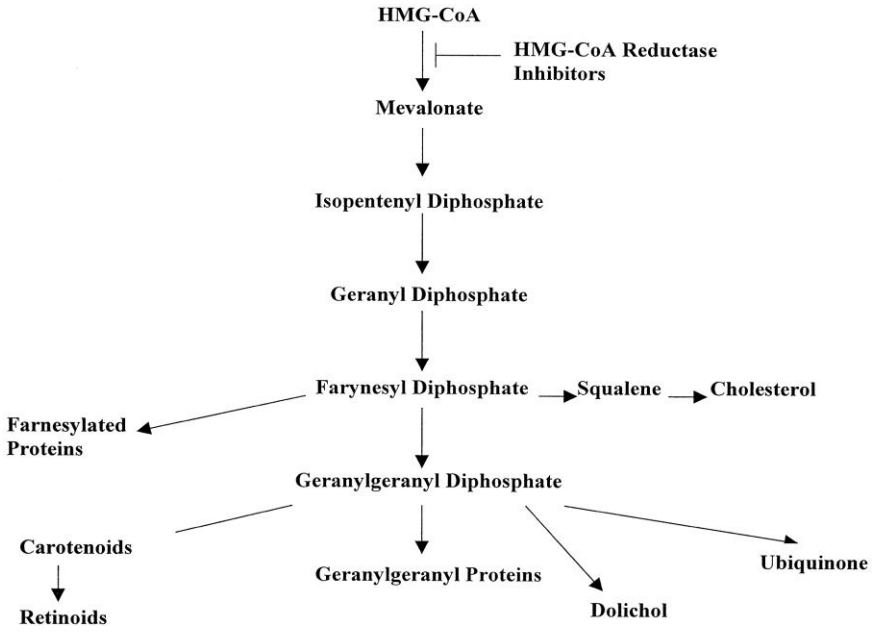


Figure 4.1 Mevalonate Pathway (Chan, Oza and Siu, 2003)

Such pleiotropic effects can range from enhanced endothelial function, anti-inflammatory, anti-oxidative, improved platelet reactivity and plaque stabilisation. Endothelial dysfunction is a precursor to atherosclerosis, owing to the disturbance in the expression in nitric oxide. Statins have been shown to have a role in modulating endothelial dysfunction via the up regulation of nitric oxidase synthase, a key enzyme responsible for nitric oxide synthesis (Laufs *et al*, 1998). This improvement of endothelial function was dependent on the inhibition of the HMGCoA reductase with a consequent reduction in the downstream metabolites. This protective effect was abolished following the introduction of mevalonate (Laufs *et al*, 1998). Furthermore, statins were shown to further augment endothelial synthase levels, by reducing the abundance of caveolin in the endothelial cells (Feron *et al*, 1999). The anti-inflammatory properties of statins are complex and are mediated via direct and indirect actions. Direct anti-inflammatory effects include allosteric binding to the LFA-1 receptor on leucocytes, thereby dampening the inflammatory response by preventing their adherence to sites of injury (Weitz-Schmidt *et al*, 2001). The indirect anti-inflammatory effects derive from the downstream reduction in mevalonate by-

products, that suppress the expression of various chemokines, in particular MCP-1 (Romano *et al*, 2000).

Of the myriad of effects, plaque stabilisation is one of the integral protective properties which are conferred by statins (Cannon *et al*, 2004). This feature is afforded by the reduction in the central lipid core, but also by a suppression of the extracellular matrix proteins concerned with remodelling, which are secreted by the macrophages. The co-incubation of atorvastatin with mouse peritoneal macrophages was shown to reduce matrix metallopeptidase-9 (MMP-9) by 40%, which persisted despite the introduction of an MMP stimulator (Bellosta *et al*, 1998). The addition of mevalonate reversed this observation, suggesting the suppression of the downstream products of mevalonate is critical for mediation of pleiotropy (Bellosta *et al*, 1998).

Statins represent one of the most commonly prescribed drug classes in the UK (Trusler, 2011). The protective effects are constantly being explored and expanding. By the same token, controversy surrounding their use is increasing, with links to the development of diabetes (Ridker *et al*, 2012; Sattar *et al*, 2010)

4.2 Aim

The aim of this study was to investigate the effect of statin therapy on lipoproteomic cargo using quantitative mass spectrometry. Exploring the modulation of lipoprotein associated proteins, may provide a greater insight into the protective/deleterious effects of statins. As statins are the cornerstone of primary and secondary prevention of CAD, characterisation of their role in lipoprotein remodelling remains unknown.

4.3 Methods

4.3.1 Materials and reagents

All materials and reagents that were used are described in Chapters 2 & 3.

4.3.2 Patient recruitment

Patient samples were donated by Dr Pankaj Gupta. Hypercholesterolemic patients (n=11) fulfilling the inclusion criteria were recruited into this prospective observation cohort study. Patients were recruited from lipid clinic from a single UK centre. Written informed consent was sought from all patients. This study complied with the declaration of Helsinki and was approved by the local ethic committee. Information regarding patients' medical history and medication history was obtained. Patients were commenced on statin therapy for primary prevention of CAD. Patients were fasted overnight prior to blood sampling. Blood was withdrawn at baseline and again after a minimum of 2 months of statin therapy. The total cholesterol, HDL, LDL and triglycerides were analysed on a Roche analyser.

4.3.3 Sample Preparation

The sample preparation is described in 3.3.4.6. All samples were prepared in a single batch. Sample preparation was performed blinded to the clinical characteristics of the patients. A quality control (QC) was generated for assessing the platform performance of the MS. The QC comprised of a pooled sample, which used 10 µl of plasma from each patient sample. The QC was injected at a regular interval to ensure platform consistency for the duration of the study.

4.3.4 Liquid chromatography coupled with high definition mass spectrometry

The samples were analysed using a data independent acquisition mode enabled with IMS on a nano UPLC coupled with to a Waters G2S Q-ToF MS (Waters Ltd.). This configuration is known as high definition MS^E or $HDMS^E$. Scouting injections of the samples were performed in MS^E (absence of mobility) mode to enable protein quantification. The volumes of the samples were adjusted, such that each injection delivered 2 µg on the column to ensure comparison between sample injections. Triplicate injections were performed for each sample. The samples were analysed in two batches. The samples were injected over a 110 minute

gradient as described previously in HDMS^e mode with ion mobility separation enabled.

4.3.5 Protein identification

Protein identification was performed using Protein Lynx Global server (PLGS) 3.0. Various thresholds were evaluated using PLGS thresholds inspector (Sourceforge). The data was analysed using a stringent FDR of 1%.

4.3.6 Protein Expression

Differential protein expression between the pre-statin and the on-statin group were analysed using Progenesis Qi software (Nonlinear Dynamics [Newcastle, UK]). The optimal settings for ion detection were 150-10-750 for the low energy, elevated energy and threshold counts respectively. Raw data files were uploaded onto the software and automatic alignment of total ion chromatograms was chosen against an automatically assigned reference run. A fixed modification of carbamidomethylation and a variable modification of oxidation M were selected for protein identification. A stringent FDR of less than 1 % was chosen. For peptide matching 2 or more fragments were required. 5 fragments were required for protein identification and 2 peptides required for protein identification. For protein quantification, Hi-3 was used. Triplicate injections of the same sample were analysed for all patients.

4.3.7 Statistical analysis

The samples were analysed in triplicate on the Water G2S MS. Protein intensities between the paired samples were analysed using the student paired t-test (two tailed). The relationship between two continuous variables was analysed using Spearman Rho correlations. A p value below 0.05 was deemed statistically significant.

4.4 Results

4.4.1 Clinical Characteristics

The baseline characteristics of the cohort are presented in Table 4.1. The mean age for the cohort was 54 ± 10 years, with a female predominance (66.6 %). The vast majority of the cohort had a family history of cardiovascular disease (90.9 %), with under a third of patients having diabetes (27.27%). Two patients were current smokers and one patient was a former smoker. The mean body mass index (BMI) for the cohort was 28.36 kg/m^2 ± 3.72. The mean HbA1c, a measure of glycaemic control over the preceding two months, was 5.6 % ± 0.71. As expected, there was a significant reduction in both total cholesterol (7.48 mmol/L ± 2.94 vs. 5.11 ± 0.90; P=0.0006) and LDL-C levels (4.62 mmol/L ± 2.53 vs. 2.69 ± 0.89; P= 0.0003) before and after statin therapy respectively, Table 4.2. No significant change in the HDL-C or triglyceride levels was observed between the baseline samples and the on statin samples.

Table 4.1 Baseline clinical variables for the 11 hypercholesterolemia patients entered into the study. Data expressed as mean ± standard deviation or percentage and number. CVD, cardiovascular disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HBA1c, glycosylated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein.

Clinical variables	Mean ± SD
Age	53.6 ± 9.91
Females (%)	66.6 (n=7)
Family history of CVD (%)	90.9 (n=10)
Diabetes mellitus (%)	27.3 (n=3)
Current smoker	18.2 (2)
Former smoker	9.1 (1)
SBP (mmHg)	137 ± 14.31
DBP (mmHg)	85 ± 10.68
BMI (kg/m^2)	28.36 ± 3.72
HbA1c	5.6 ± 0.71
Total Cholesterol (mmol/L)	7.48 ± 2.94
LDL (mmol/L)	4.62 ± 2.53
HDL (mmol/L)	1.61 ± 0.50
Triglycerides (mmol/L)	2.95 ± 3.36

Table 4.2 The effect of statins on the lipid profiles of the 11 hypercholesterolemia patients. Data expressed as mean ± standard deviation.

	Pre statin	On statin	P value
Total Cholesterol (mmol/L)	7.48 ± 2.94	5.11 ± 0.90	0.0006
LDL (mmol/L)	4.62 ± 2.53	2.69 ± 0.89	0.0003
HDL (mmol/L)	1.61 ± 0.50	1.58± 0.28	0.9158
Triglycerides(mmol/L)	2.95 ± 3.36	2.45 ± 1.70	0.5952

4.4.2 Platform reproducibility

The pooled QC was analysed at the beginning, middle and at the end of each batch. There was no significant difference in mean protein hits for the QCs performed in the first and second batches (131.33 ± 1.53 vs. 135.33 ± 4.16; P=0.12) respectively, injections indicating reliable data acquisition.

Furthermore, there was no significant difference in the mean number of peptides identified for the QC injections, between the first and second batches (2170.33 ± 15.31 vs. 2193 ± 74.71; P=0.66) respectively, Figure 4.2. Both observations suggest that the performance of platform was consistent and reliable, ensuring valid data acquisition of the clinical samples. The number of peptides identified for a selection of proteins of varying concentration, were similar between the two batches, reinforcing consistent data acquisition Figure 4.3.

The chromatograms for the QC injection performed in the two batches are presented in Figure 4.4. The ion with the highest intensity was chosen for evaluating the drift across the QC samples. The extracted ion chromatograms for ion 575.29 m/z yielded a mean retention of 36.4 minutes (SD± 0.20), indicating minimal drift across the injections.

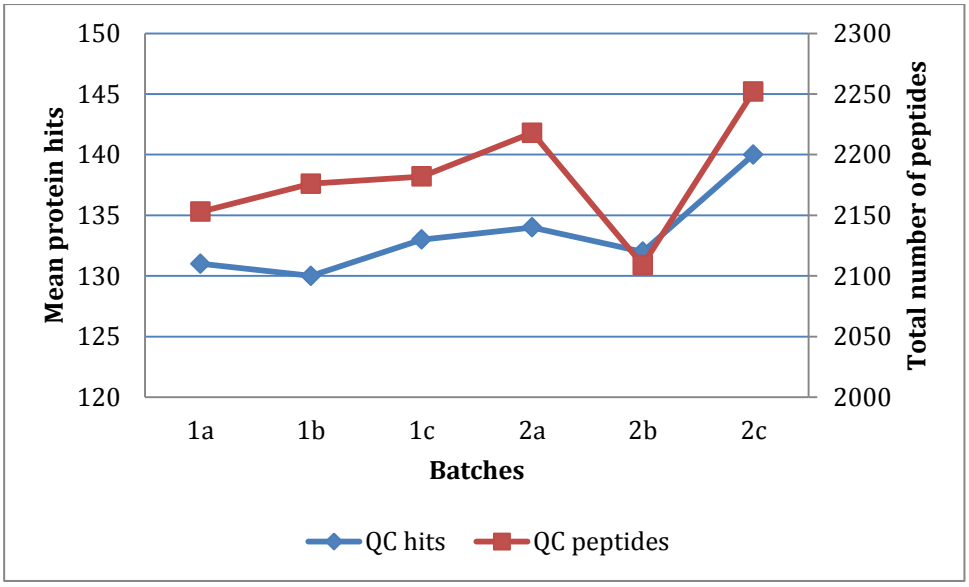


Figure 4.2 Protein hits and total number of peptides identified for the QC injection performed prior (a), during (b) and at the end (c) of each respective batch. Protein hits and the total number of peptides for the injection were consistent during the samples injections indicating reliable data acquisition.

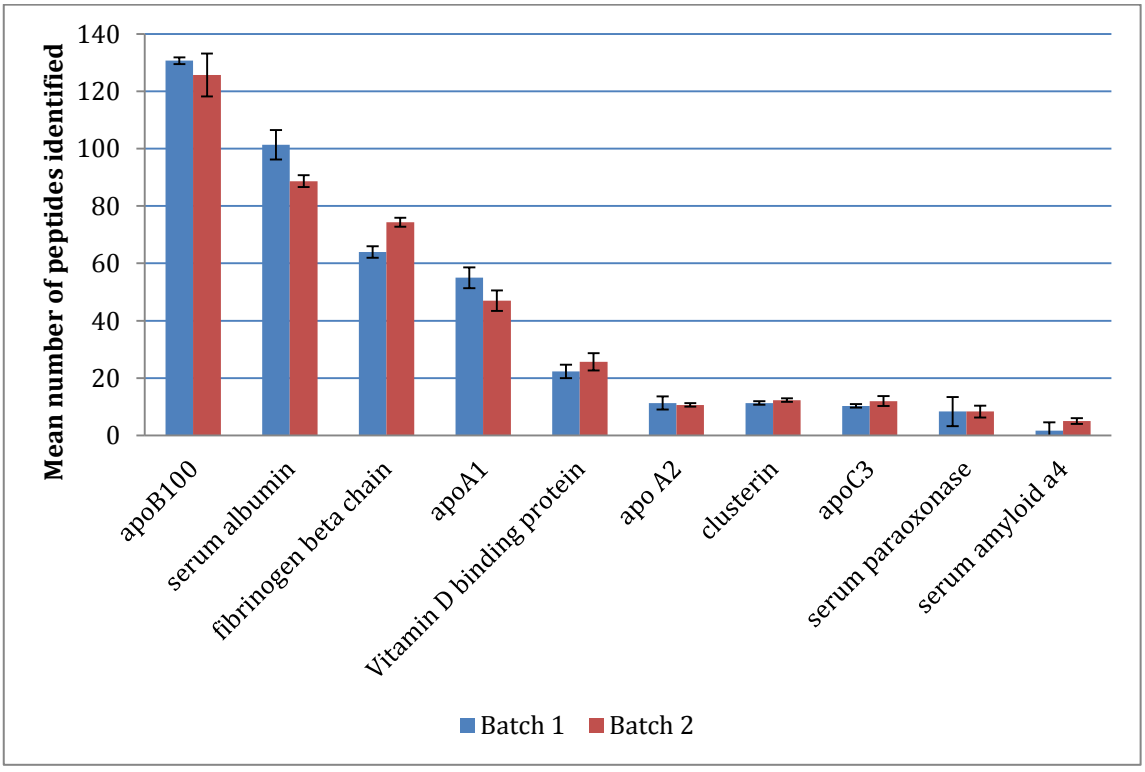


Figure 4.3 Bar chart showing the total number of peptides identified for randomly selected proteins derived from the QCs analysed in batch 1 and batch 2. The number of peptides per protein is very consistent. Bars representing the mean and the error bars represent.

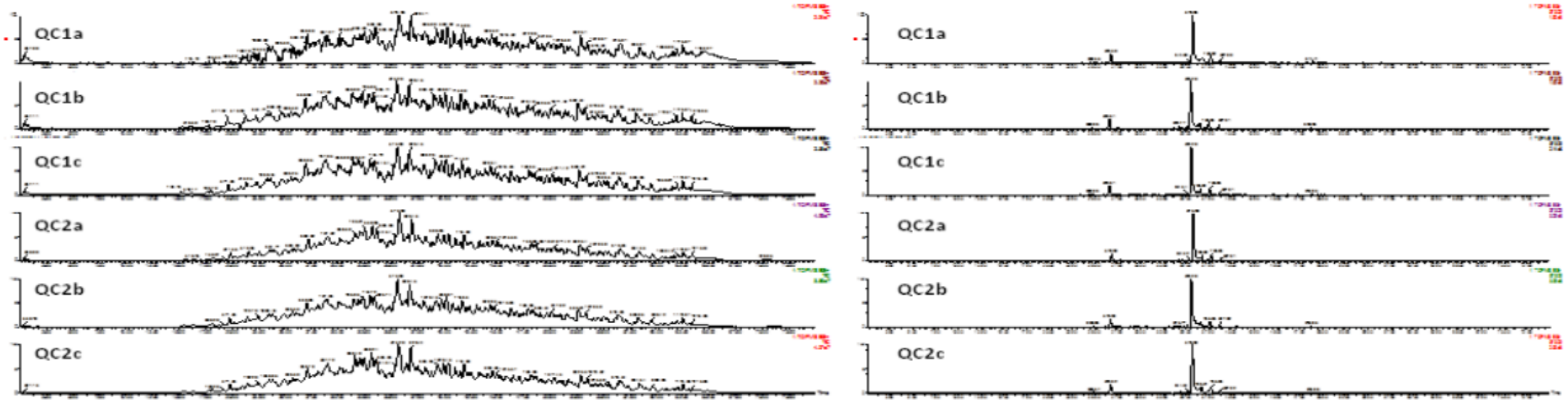


Figure 4.4 The chromatograms for the QC injections run at the beginning, middle and end of each of the two batches. The extracted ion chromatogram for ion with m/z of 575.29 identified in each of the QC injections performed at the beginning, middle and end of the 2 batches.

4.4.3 Protein identification

The optimal high energy, low energy and intensity thresholds were 150-10-750 respectively. The mean protein hits for the pre statin and on statin samples are presented in Figure 4.5. There was no significant difference in the number of protein hits between the pre statin and the on statin samples (133.36 ± 7.33 vs. 142.56 ± 11.04; P=0.066) respectively, with any variation likely to represent biological variation in light of the consistent data acquisition. The CV for the triplicate protein hits for each sample demonstrated good precision with CVs lower than 15%, Figure 4.6. In addition, there was no significant difference in the CV for the protein hits between the two sample sets (P=0.32).

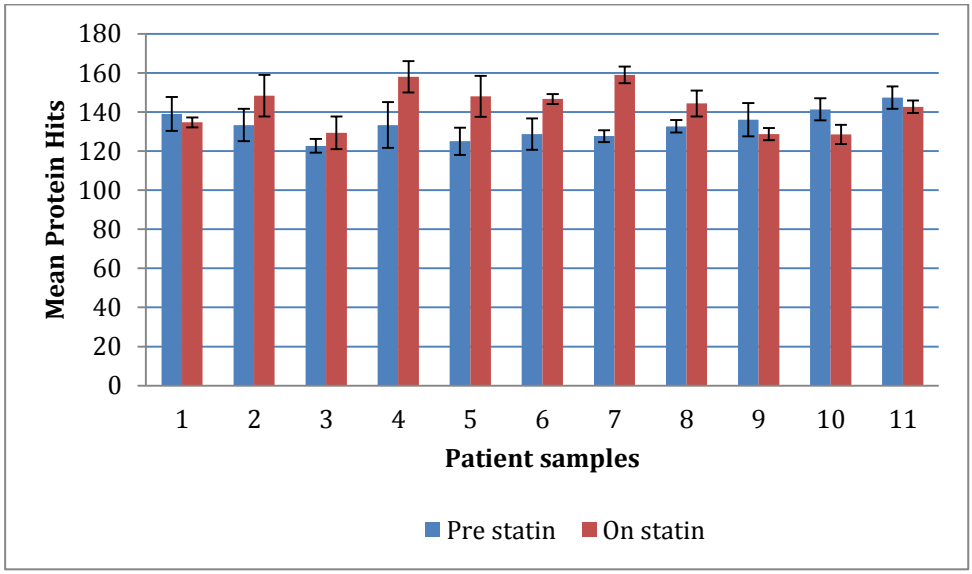


Figure 4.5 Bar chart showing the protein hits for the pre statin and on statin groups. N=3, data expressed as mean ± standard deviation.

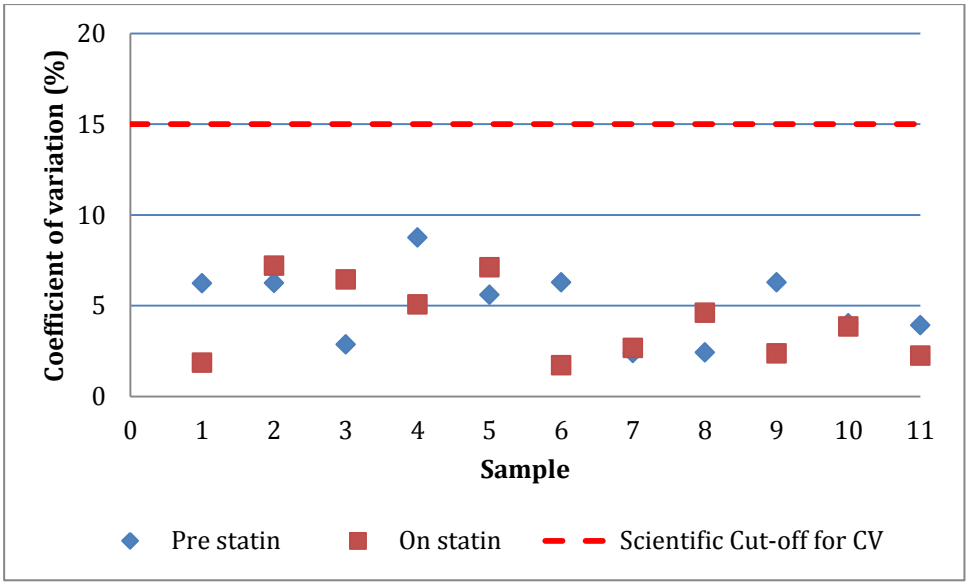


Figure 4.6 Scatter graph showing the coefficient of variation (CV) in protein hits before and during statin therapy.

4.4.4 Expression Analysis

218 proteins were identified in all samples using a stringent FDR of 1 %. Thirty three proteins demonstrated significant differential expression; 17 proteins showing enrichment and 16 proteins showing depletion with statin therapy, Table 4.3 and Figure 4.7.

Table 4.3 Significant differentially expressed proteins in the 11 hypercholesterolemia patients treated with statin therapy. Data values expressed as mean ± standard deviation.

Protein	Mean pre statin protein intensities	Mean on statin protein intensity	Fold change (On statin/Pre statin)	Paired T Test
Vinculin	6207.35 ± 1801.68	10724.13 ± 2645.20	1.73	1.23E-05
C-type lectin domain family 11 member A	38868.34 ± 9807.32	27621.12 ± 7773.74	0.71	7.93E-05
Tenascin-X	441.44 ± 157.28	258.99 ± 81.01	0.59	9.54E-05
Apolipoprotein B-100	1838441.79 ± 489760.63	1281279.12 ± 237279.17	0.70	5.81E-04
Ig kappa chain V-I region Wes	611.20 ± 243.31	1336.62 ± 571.07	2.19	6.72E-04
Proteoglycan 4	193.86 ± 94.52	75.31 ± 55.10	0.39	2.11E-03
Beta-Ala-His dipeptidase	5634.84 ± 1300.66	4053.12 ± 1100.80	0.72	0.002
Thiosulfate sulfurtransferase/rhodanese-like domain-containing protein 1	451.89 ± 95.27	626.05 ± 185.27	1.39	0.003
Coagulation factor IX	11264.99 ± 1539.28	13118.61 ± 1335.11	1.16	0.003
Complement factor D	2167.47 ± 847.77	1445.68 ± 467.90	0.67	0.006
Transgelin-2	847.53 ± 297.31	1459.72 ± 552.10	1.72	0.007
Integrin beta-3	369.33 ± 267.34	898.49 ± 575.01	2.43	0.009
Peroxiredoxin-2	1151.34 ± 473.31	1613.28 ± 503.78	1.40	0.009
Apolipoprotein F	12065.33 ± 4635.36	8294.98 ± 3061.68	0.69	0.010
Serum amyloid A-1 protein	3327.73 ± 491.23	3982.21 ± 709.35	1.20	0.010
Tropomyosin alpha-4 chain	2317.63 ± 700.53	3050.21 ± 863.25	1.32	0.012
Ig kappa chain V-III region NG9 (Fragment)	1527.42 ± 788.40	876.06 ± 256.42	0.57	0.013
Hepatocyte growth factor-	20330.15	18618.58	0.92	0.017

like protein	± 3576.48	± 3046.76		
Tubulin beta-1 chain	4353.36 ± 917.01	5411.45 ± 1299.31	1.24	0.017
Apolipoprotein E	245913.71 ± 95540.45	167367.50 ± 32987.78	0.68	0.023
Profilin-1	13420.56 ± 4060.73	16260.59 ± 3695.46	1.21	0.026
Uncharacterized protein CXorf38	3423.89 ± 742.27	4311.54 ± 582.37	1.26	0.027
Fructose-bisphosphate aldolase B	163.86 ± 139.62	346.32 ± 219.95	2.11	0.028
Angiotensinogen	16784.55 ± 7539.09	11489.75 ± 2346.78	0.68	0.028
Ig heavy chain V-III region GAL	10681.90 ± 2111.40	13065.41 ± 2823.56	1.22	0.031
Acyl-CoA dehydrogenase family member 10	86.85 ± 29.44	125.44 ± 35.62	1.44	0.037
Ig lambda chain V-III region LOI	2975.92 ± 1268.65	4113.82 ± 1866.63	1.38	0.041
Carboxypeptidase N subunit 2	9467.83 ± 2458.04	6984.96 ± 2045.02	0.74	0.042
Ig kappa chain V-III region POM	32.66 ± 43.28	54.40 ± 55.88	1.67	0.043
Monocyte differentiation antigen CD14	1456.22 ± 594.08	1094.74 ± 265.04	0.75	0.044
Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H4	108.33 ± 276.44	93.21 ± 271.05	0.86	0.047
Transforming growth factor beta regulator 1	4988.78 ± 1519.58	3961.08 ± 801.06	0.79	0.049
Ig lambda chain V-III region SH	8917.08 ± 3412.49	7385.94 ± 2138.33	0.83	0.049

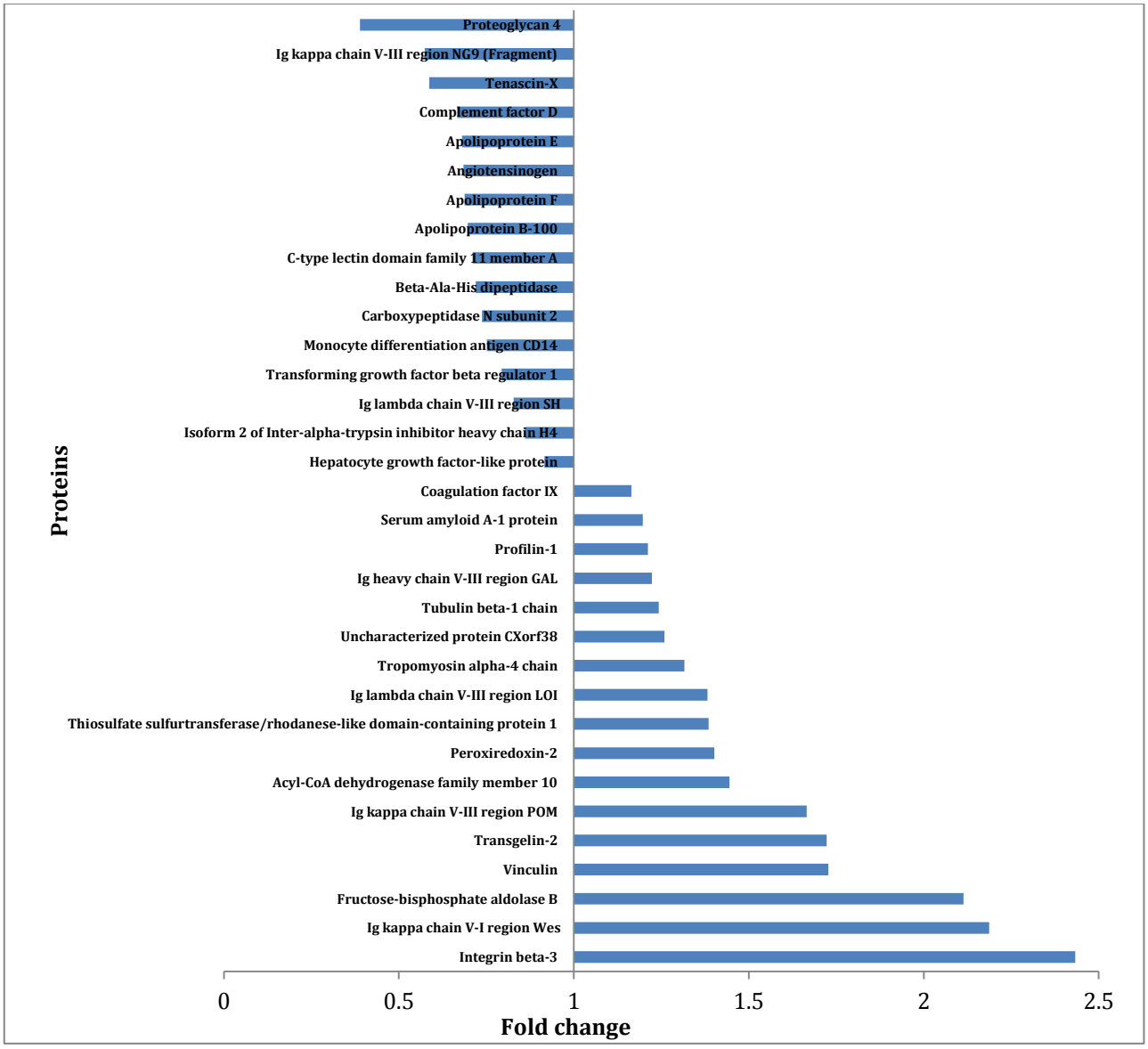


Figure 4.7 Bar chart showing the fold change in protein intensities between the pre statin and the on statin groups. Fold change >1 indicated protein enrichment in the statin group. Fold change <1 indicates protein depletion in the statin group. Data derived for triplicate analysis in Progenesis QI for Proteomics. Data subject to paired t-test analysis.

4.4.5 Correlations

There was a significant correlation between the laboratory determined LDL levels and the MS derived apoB100 intensities (spearman's rho 0.816; P<0.0001), Figure 4.8. Consistent with the mechanism of action for statin, the apoB100 intensities were significantly lower in the statin group compared with the pre statin group (1838441.79 ± 489760.63 vs 1281279.12 ± 237279.17; P=5.81E-04), Figure 4.9.

In addition, there was a significant relationship between the laboratory determined HDL levels and the MS derived apoA1 intensities (Spearmans' rho 0.489; P=0.040), Figure 4.10. Statin therapy had no significant effect on apoA1 intensities compared to baseline (3921897 ± 1066873 vs. 4514732 ±1165876; P=0.094) respectively, which is consistent with the HDL-C findings.

The concordance between the lab values of LDL and HDL and the MS protein intensities for apoB100 and apoA1 respectively, suggests that iterative processes behind progenesis QI are reliable.

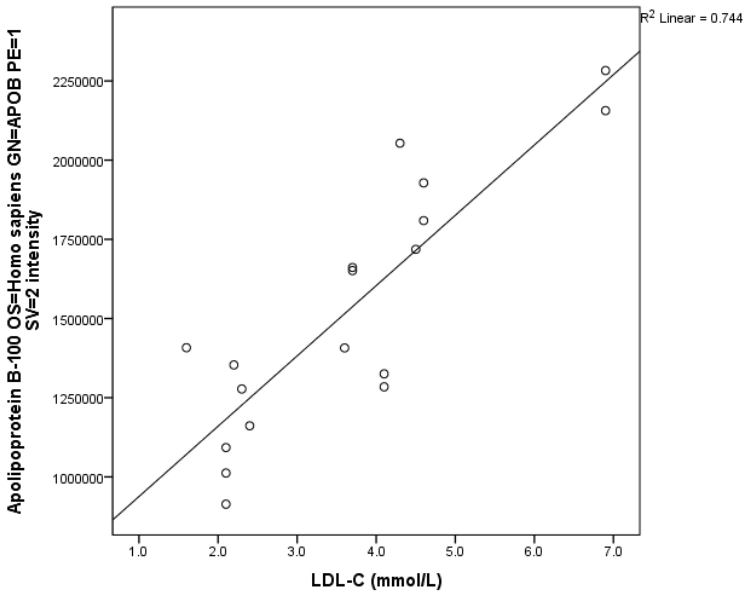


Figure 4.8 Scatter graph showing the relationship between LDL-C levels and apoB100 intensities (spearman's rho 0.816; P<0.0001)

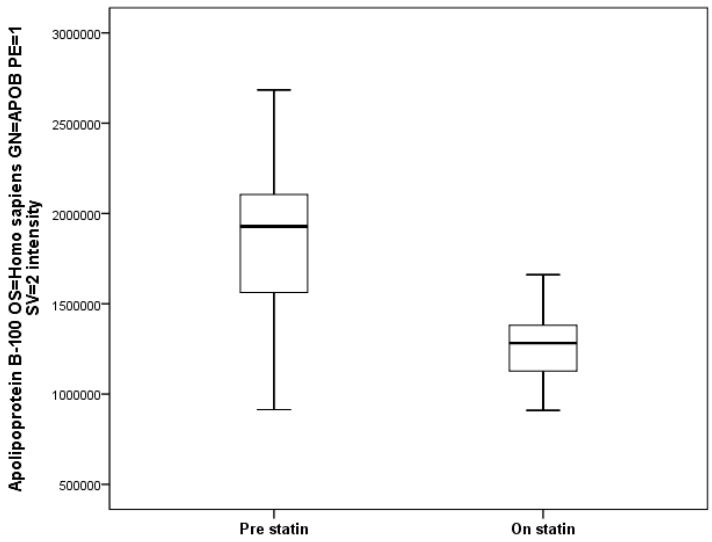


Figure 4.9 Boxplot showing the apoB100 intensities between the pre statin and on statin samples (P<0.001).

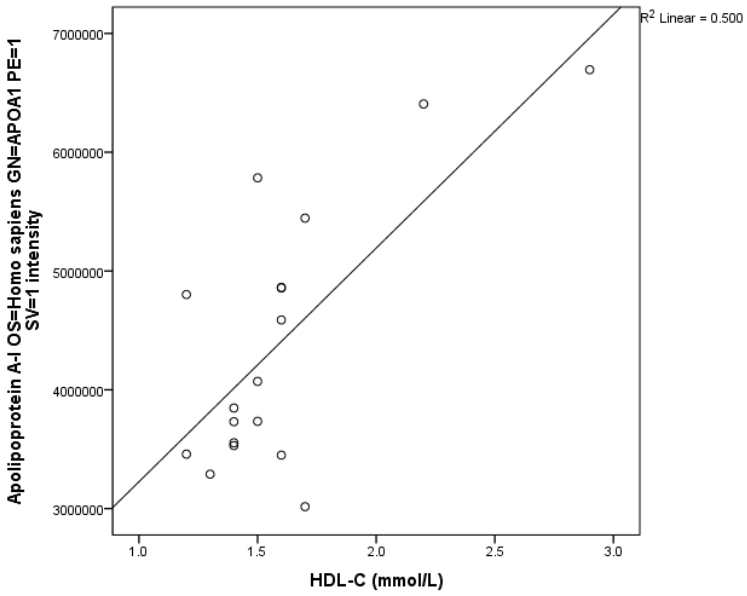
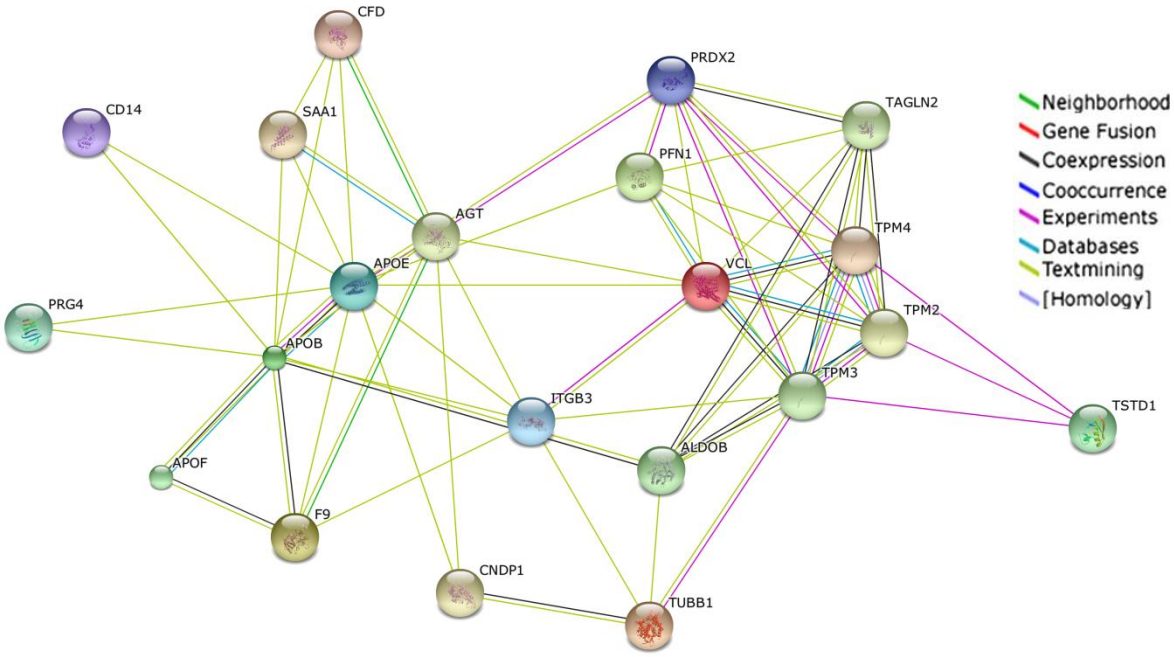


Figure 4.10 Scatter graph showing the relationship between HDL-C levels and apoA1 intensities (Spearmans' rho 0.489; P=0.040)

4.4.6 Pathway analysis

The proteins that demonstrated significant differential expression with statin therapy were analysed using STRING software to determine protein interactions Figure 4.11A. Cluster analysis revealed 3 distinct protein clusters, Figure 4.11B. This highlights the diverse interactions between different proteins in mediating an array of cellular functions.

A



B

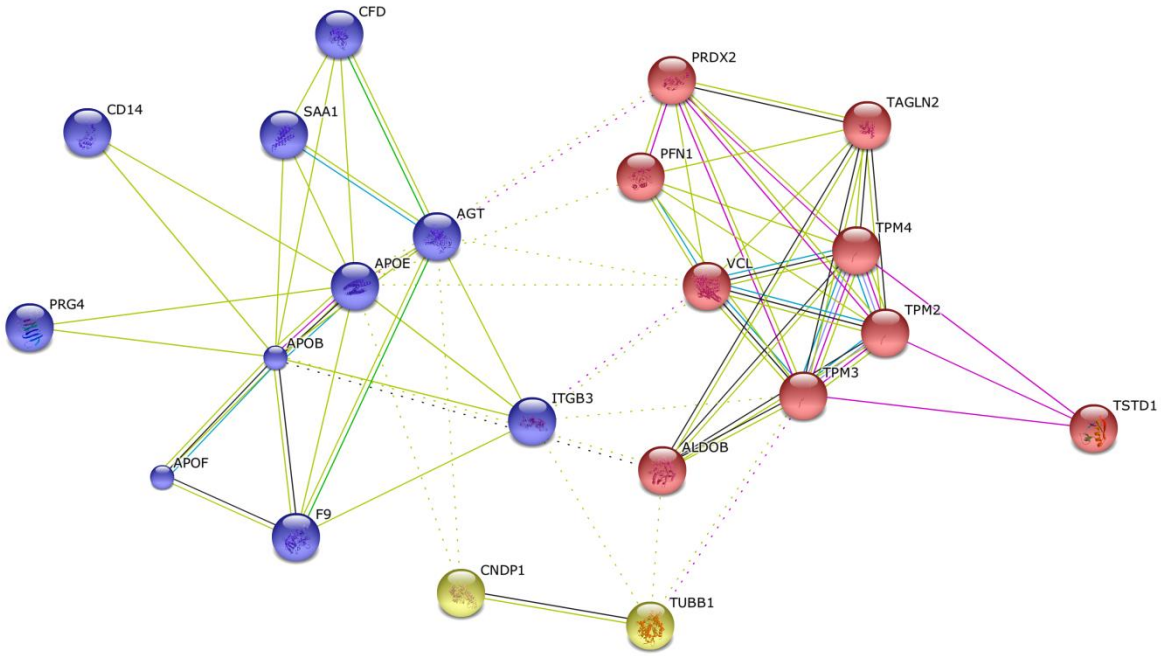


Figure 4.11 Protein-protein interaction networks visualised by STRING. (A) Protein interaction networks of the dysregulated proteins with statin therapy. (B) Protein network clustering. The different colours represent the different levels of evidence for the interactions

4.5 Discussion

The survival benefits conferred by statins in primary and secondary prevention of cardiovascular disease are well founded. It is evident that statins mediate these benefits primarily via the inhibition of the HMGCoA reductase pathway, which is concerned with the biosynthesis of cholesterol and various other metabolites which are integral in the cell growth and cell death. The depletion of mevalonate and the consequent depletion of the downstream by-products, have a knock-on effect on their availability for participation in cellular pathways, which are likely to contribute to the pleitropic effects of statins.

4.5.1 Vinculin

In this study, vinculin was found to be up regulated by statin therapy compared to the baseline (10724.13 ± 2645.20 vs. 6207.35 ± 1801.68; P=1.23E-05) respectively. Vinculin is a cell adhesion protein, which stabilises the actin filaments

to the plasma membrane and therefore contributes to the cytoskeletal architecture. It was reported that normal human umbilical vein endothelial cells (HUVEC) had negligible vinculin expression, however their levels increased with endothelial dysfunction induced by thrombin (van Nieuw Amerongen *et al*, 2000). Statin therapy was shown to redress the balance in favour of endothelial function by preventing the transmigration of the LDL particles, but also by reducing the formation of the stress fibres and the focal adhesion proteins (van Nieuw Amerongen *et al*, 2000). Hypercholesterolemia is a known risk factor for CAD, it is therefore plausible that such patients may have a degree of endothelial dysfunction with elevated vascular levels of vinculin and stress fibres. Statin therapy is likely to have resulted in a reorganisation of the cytoskeleton to restore normal function, with consequent leakage of vinculin into plasma, accounting for the increased levels. An alternative explanation for the increased levels of vinculin with statin therapy may be due to indeterminate disruption of the cytoskeleton via the depletion of the isoprenoid intermediates as observed in cultured cardiac fibroblasts (Copaja *et al*, 2012). It was revealed that the organised actin filaments in cultured cardiac fibroblast were disrupted along with reduced cell counts after 24 hours of statin incubation (Copaja *et al*, 2012). This observation was reduced by the introduction of mevalonate and isoprenoid intermediaries. Furthermore, it was shown that the membrane adhesion complex which included vinculin was disrupted, resulting from a redistribution from its native position in the plasma membrane to the cytoplasm (Copaja *et al*, 2012). Such disruption induced by statin therapy may contribute to cell death from reduced cell signalling and account for leakage of vinculin into plasma, in a similar way to that of statin induced myopathy (Copaja *et al*, 2012).

4.5.2 Adipsin

In this study, adipsin also known as complement factor D, was found to be down regulated with statin therapy compared to the pre statin group (1445.68 ± 467.90 vs. 2167.47 ± 847.77; P=0.006), respectively. Adipsin is highly expressed in the adipose tissue and is involved in the alternative complement pathway (White *et al*, 1992). It has recently been suggested that adipsin may be a novel adipokine, as Lo

et al showed that adipsin null mice exposed to a high fat diet for a prolonged duration of 16 weeks, had a significant disturbance in glucose homeostasis with lower fasting insulin levels, compared with wild type mice (Lo *et al*, 2014). Interestingly, in a murine model of diabetes, an injection of adipsin improved fasting glucose levels and increased basal insulin levels, compared to control diabetic mice. In humans, adipsin expression in the adipose tissue was significantly lower in diabetic patients with β cell failure, compared to those without β cell failure. Furthermore, a similar trend was observed for plasma levels of adispin, which were significantly lower in the diabetic patients with β cell failure compared to those without β cell failure. The other adipokines did not demonstrate any differential expression between the two sets of patients (Lo *et al*, 2014). It was suggested that in metabolic syndrome, despite the initial reduction in adipsin expression in the adipose tissue, the overall expansion of this compartment may maintain sufficient levels of adispin, however as adipose dysfunction progresses, compensation may become insufficient and dysglycaemia ensues (Lo *et al*, 2014). The suppressive effect of statins on adipsin levels may shed light on the controversial link between statin use and new onset diabetes. The association was extensively explored in a sub analysis of JUPITER study, which revealed that statin treated patients with at least one risk factor for diabetes (metabolic syndrome, impaired fasting glucose, HBA1c > 6% or BMI$\geq$30kg/m^2) had a 28% increased risk of developing diabetes compared to those without risk factors(Ridker *et al*, 2012). By stark contrast, statins conferred a 39% reduction in CVD events in this cohort, negating the risk of developing diabetes (Ridker *et al*, 2012). This work corroborated an earlier meta-analysis, which reported that statins increased the overall risk of diabetes by 9%, but this was offset by large reductions in coronary events (Sattar *et al*, 2010). The common perception is that the association between statins and diabetes may be confounded by a population already at increased risk of developing the disease, and that the introduction of the drug may simply bring the diagnosis earlier (Shah and Goldfine, 2012). The simplicity of a single pathway mediating diabetes is very attractive; however, the aetiology of diabetes is multifactorial and is therefore likely to be a culmination of many interacting and competing pathways that contribute to insulin resistance and dysgylcaemia.

4.5.3 Peroxiredoxin-2

In this study, peroxiredoxin-2 was up regulated with statin therapy compared to the baseline samples (1613.28 ± 503.78 vs. 1151.34 ± 473.31; P=0.009) respectively. Peroxiredoxin-2 belongs to a family of thiol dependent antioxidants, which are expressed in the cytoplasm, mitochondria, nuclei and plasma (Wood *et al*, 2003). Peroxredoxin-2 possesses 2 cysteine residues which are vital for detoxification of hydrogen peroxide (Chae *et al*, 1994). This is a powerful antioxidant which was recently highlighted in murine knockout models, whereby apoE and peroxiredoxin-2 knockout mice displayed greater atherosclerotic disease compared to control apoE null mice (Park *et al*, 2011). Furthermore, peroxiredoxin-2 deficiency resulted in greater inflammatory cell recruitment and infiltration mediated by increased expression of the chemokine MCP-1 and the adhesion molecule VCAM-1 (Park *et al*, 2011). The up regulation of peroxiredoxin-2 by statin therapy may therefore confer protection against oxidative stress.

4.5.4 Transgelin 2

In this study transgelin 2 intensity levels were found to be higher in the statin group compared to the pre statin group (1459.72 ± 552.10 vs. 847.53 ± 297.31; P=0.007). This protein belongs to the calponin family, which regulates the cross linking of the actin filaments involved in cell motility. Lovastatin was recently shown to reduce motility and tube formation in HUVECS after 24hours of incubation (Xiao *et al*, 2012). Furthermore, it was revealed that lovastatin reduced myosin light chain phosphorylation, which is integral in cell motility (Xiao *et al*, 2012). Proteomic exploration of the HUVECS cell lysate, revealed an enrichment of trangelin 2 with statin exposure (Xiao *et al*, 2012). This mechanism was linked to an inhibition of the Rho kinase pathway, as rho kinase inhibitors had a similar effect on increasing transgelin 2 levels, inferring a possible chemopreventative role for statins (Xiao *et al*, 2012). In-vitro studies using statin have demonstrated desirable antiproliferative and anticancer effects, however in vivo studies have suggested otherwise. In the PROSPER study, the efficacy of pravastatin treatment in an elderly cohort was examined (Shepherd *et al*, 2002). The primary endpoint of death, MI and stroke was reduced by 15% in the treated group, however this

group endured a 15% increase in new cancer diagnosis compared to the placebo group. Incorporation of this finding into a metaanalysis of all pravastatin trials eliminated any association with cancer and was labelled as a chance finding (Shepherd *et al*, 2002). One study that was included in the metaanalysis was the CARE trial, which showed that, despite an overall neutral effect on all cancer, new breast cancer diagnosis was higher in the pravastatin group than the placebo (Sacks *et al*, 1996). The long term effects of statin therapy on cancer incidence necessitates longer follow up, to explore this relationship further and to bridge the discrepancy between laboratory and clinical observations.

The depletion of the mevalonate by-products and subsequent inhibition of the Rho pathway is likely to mediate increased levels trangelin 2 levels, which exert a protective role when considered in isolation (Xiao *et al*, 2012).

4.5.5 C-type lectin domain family 11 member A

C-type lectin domain family 11 member, also known as stem cell growth factor (SCGF), was found to be down regulated with statin therapy (27621.12 ± 7773.74 vs. 38868.34 ± 9807.32; P=7.93E-05). This protein is a known stimulator of haemopoetic progenitor cells (Hiraoka *et al*, 2001).

4.6 **Verification**

The identification of significant differentially expressed proteins between the pre statin and statin group using mass spectrometry represents one rung on the biomarker discovery ladder. The next step requires verification of the dysregulated proteins in the clinical samples, to confirm or refute the MS findings. Due to limitations in time, only one dysregulated protein was taken forward for verification using immunoassays. The potential mechanistic link between statin use and new onset diabetes would represent a huge scientific breakthrough, so for this reason adipsin was selected for verification.

4.6.1 Materials and reagents

All materials and reagents are as described in Chapter 2. Mouse anti-human adipsin (capture) and biotinylated goat anti-human adipsin (detection) antibodies were purchased from R & D systems (Minneapolis, US).

4.6.2 Method

In this study quantification and ratification of the adipsin findings was sought using a commercially available immunoassay. The procedure was as follows:

Day 1

- Microtiter wells were coated with the 400 ng coating antibody (R & D systems) and incubated overnight.

Day 2

- Wash wells with solution C
- Add 150 µL of blocking agent to each well (0.5 % BSA and azide)
- Incubate for 2 hrs on shaker
- Wash well with Solution B

- Add standards (R & D systems) to wells, top standard was 200 pg per well
- Serial 2-fold dilution.
- To 1 µL of plasma add 1999 uL of ILMA.
- Remove 20 µL and add to well
- Incubate at room temperature on the shaker overnight.

Day 3

- Wash wells with solution B
- Add 200 ng of biotinylated goat antibody (R & D systems) to each well
- Incubate for 2 hours at room temperature
- Wash well with solution B
- Add 100 µL of streptavidin to each well
- Incubate for 1.5 hours in the dark at room temperature
- Wash wells with solution B
- Read plate on MLX Luminometer (Berthold, Germany).

4.6.3 Results

The assay was performed according to the manufactures instructions. The ideal plasma concentration was assessed using a serial 2 fold plasma dilution, with the top standard starting at 100 nL per well. A plasma dilution of 20 nL was chosen as the optimal concentration as it was within the range of the standard curve Figure 4.12.

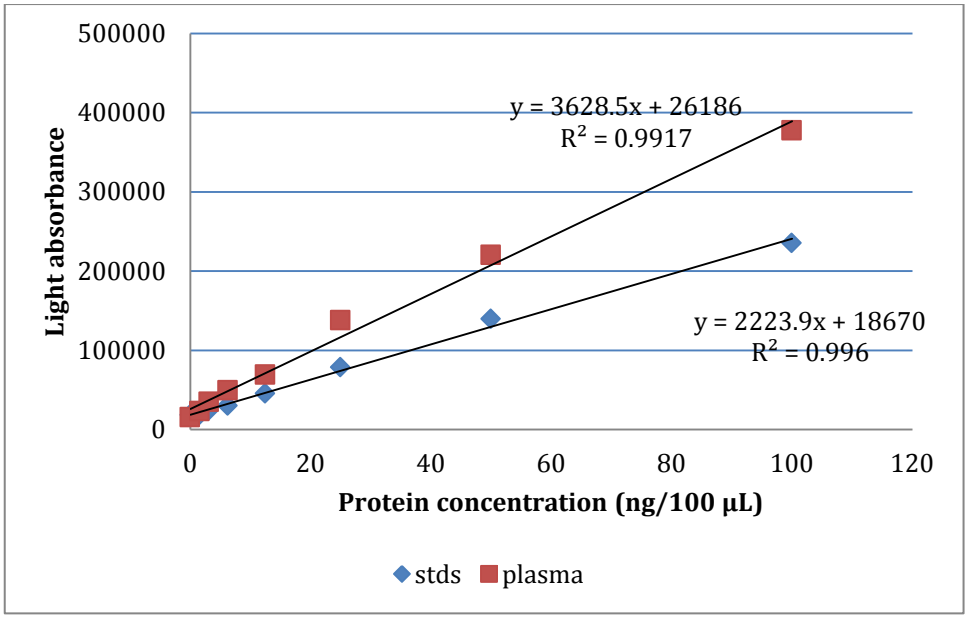


Figure 4.12 Serial dilutions for adipsin standards and plasma using commercially available adispin assay

Plasma samples from the patients with hypercholesterolaemia were assayed according to the protocol described in section 4.6.2. Below is the standard curve for the adipsin assay Figure 4.13.

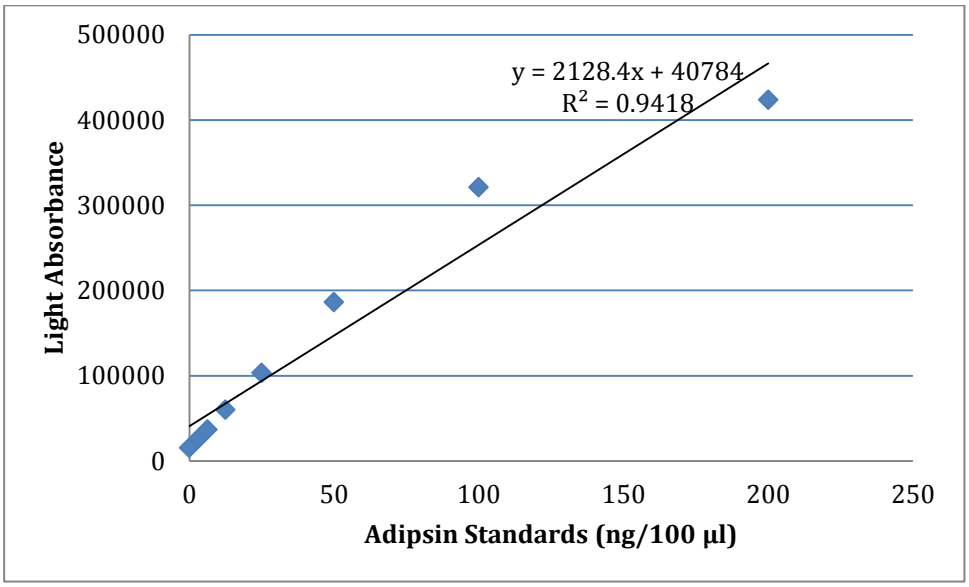


Figure 4.13 Standard curve for the adipsin assay.

Using a commercially available adipsin immunoassay, there was no significant difference in plasma adipsin concentrations between the pre statin group and the on statin group (1.50 ± 0.30 vs. 1.55 ± 0.48; P=0.62 respectively). This contrasts with the MS results, which supported a depletion in adipsin intensities with statin therapy compared to the prestatin groups (1445.68 ± 467.90 vs. 2167.47 ± 847.77; P=0.0056 respectively). There was no significant correlation between the immunoassay and the MS results (Spearman's rho 0.190; P=0.396). Furthermore, there was no significant correlation between the prestatin immunoassay and prestatin MS results (Spearman's rho 0.009; P=0.979), however there was a trend towards significance for the on statin samples between the two different modalities (Spearman's rho 0.600; P=0.051). The failure to replicate the MS data on plasma samples was a concern. This raised 2 key issues;

1. Is the Progenesis Qi software reliably identifying differentially expressed proteins?
2. Is adipsin present in varying degrees in the plasma i.e. free and unbound compared to bound?

The progenesis Qi software identified a significant reduction in apoB100 in the statin group compared with the pre statin group. This is consistent with the mechanism of action for statins. Furthermore, there was a significant correlation between the laboratory determined LDL levels and the MS derived $apoB_{100}$ intensities (spearman's rho 0.816; P<0.0001).

Statins have a negligible effect on HDL levels, in this study there was no significant difference in HDL levels between the pre statin and the on statin groups (1.61 mmol/l ± 0.51 vs. 1.58 ± 0.28; P=0.92 respectively). In keeping with this, the apoA-1 intensities were not significantly differentially expressed between the pre statin and the statin groups (4514732 ±1165876 vs. 3921897 ±1066873; P=0.094). In addition, there was a significant relationship between the laboratory determined HDL levels and the MS derived apoA1 intensities (Spearmans' rho 0.489; P=0.040). The concordance between the lab values for LDL and HDL and the MS protein intensities for apoB100 and apoA1, suggests that iterative processes behind progenesis QI are reliable.

In this study, the lipoproteins and their associated protein cargo were isolated using a novel lipoaffinity resin, with very good reliability and reproducibility. It is therefore conceivable that adipsin is carried as part of the lipoprotein cargo either on HDL or LDL and may reflect different secretory/ behavioural patterns to the unbound adipsin present in plasma. To investigate this further, the proportion of unbound adipsin and bound adipsin in plasma was determined, as this has not been previously reported.

We therefore measured the adispin concentration in the plasma before and after affinity resin incubation to determine the amount of bound adipsin. However, there was no significant difference in the bound adipsin between the prestatin and the on statin groups (17.03± 5.05 vs. 15.61±6.11; P=0.222) Figure 4.14.

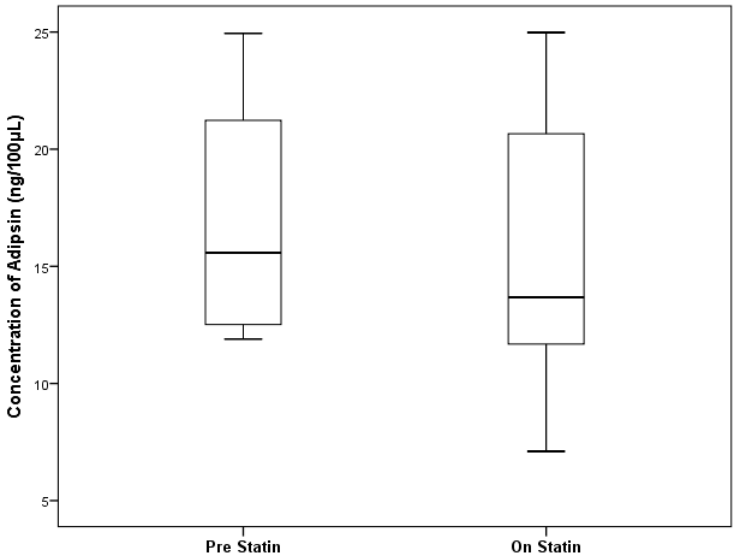


Figure 4.14 Boxplot showing the concentration of adipsin in the pre-statin and in the on-statin samples (P=0.222).

4.7 Conclusion

Statins are the cornerstone for primary and secondary prevention for cardiovascular disease. The evidence supporting their health benefits is overwhelming, however, the controversy surrounding their use is becoming the subject of media frenzy and likely to impact on patient uptake and compliance. Statins mediate both lipid dependent and lipid independent effects, which are principally protective but maybe deleterious. One such negative association is the link with new onset diabetes. In this study, adipsin a novel adipokine, which has been shown to stimulate insulin secretion, was down regulated with statin therapy using a lipoproteomic based approach. This candidate protein was selected for verification in the clinical samples, but failed to demonstrate any significant difference. The failure of the verification step may have been due to limitations in the study design, proteomic platform, bioinformatics or the immunoassay design. A potential limitation is the small study size (n=11). The proteomic platform employed in this study was previously shown to be valid and reproducible, furthermore the differences observed between the pre statin and on statin group were consistent with the clinical expectations i.e. apoA1 and apoB100. Alternatively, the immunoassay may not have the adequate sensitivity for adipsin and may be cross reacting with similar species negating any true difference.

A potential limitation in the identification of differentially expressed proteins was the lack of correction for multiple testing. The conventional use of Bonferroni correction for multiple testing may limit false positives at the expense of identifying novel proteins. Progenesis Qi software uses a false discovey rate which although is less stringent than the Bonferroni, accounts for multiple testing by adjusting the p value.

Chapter 5

5 Lipoproteomic differences between CAD patients and control subjects

5.1 Introduction

CAD is a leading cause of mortality and morbidity worldwide. Various risk scores have been devised to attempt to accurately identify high risk patients who would benefit from primary preventative measures. The original risk score was derived from the Framingham heart study, which acknowledged factors such as blood pressure, total cholesterol, smoking history and dysglycaemia, as strong determinants of prospective cardiovascular disease (Kannel, McGee and Gordon, 1976). Risk scores have evolved to encompass a social perspective alongside the conventional risk factors, to further identify high risk patients. The QRISK2 score incorporates a social deprivation score and accounts for inflammatory diseases such as rheumatoid arthritis, which are strongly influential in the aetiology of CAD (Hippisley-Cox *et al*, 2008). Furthermore, the QRISK2 which is now commonly used in primary care is more representative of the UK population, rather than the US derived Framingham risk score, as it takes into account the individual's ethnicity. In reclassification analysis, the QRISK2 was able to accurately down classify 40% of high risk individual defined by a modified Framingham risk score, into low risk categories, thereby improving resource efficiency at a time of financial constraints (Hippisley-Cox *et al*, 2008).

Despite this, the clinical utility of such tools is limited with many individuals undiagnosed for CAD. This has spurred immense interest into identifying novel disease markers that give an insight into the pathways and mechanism which are pivotal in the aetiology of atherosclerosis. One such mechanism is inflammation, which is closely related to the initiation, progression and rupture of an atherosclerotic plaque. C related peptide (CRP) is an acute phase reactant, which is closely modulated by various cytokines and its levels have been shown to be associated with underlying CAD (Ridker *et al*, 1997). In a prospective study, it was revealed that baseline CRP levels were significantly higher in individuals who later developed MI, compared to event free individuals. Compounding this association,

individuals with CRP levels in the highest tertile, had a twofold increased risk of MI, compared to those in the lowest tertile. The predictive capacity conferred by CRP was shown to be independent of the known CAD risk factors (Ridker *et al*, 1997). It was therefore suggested that CRP offered incremental and complementary prognostic information to current risk scoring parameters. This association was reinforced in another study, using a highly sensitive immunoassay for CRP measurement, in healthy male subjects followed up over 8 years. It was reported that CRP was independently predictive of first major coronary event in such individuals, lending further weight to the inflammation/atherosclerosis theory (Koenig *et al*, 1999).

The causal relationship between the lipoproteins levels and CAD, along with their involvement in various inflammatory pathways, due to their unique low abundant protein cargo, represents a potentially interesting avenue in which to identify novel biomarkers. The relatively small lipoproteome in comparison to the plasma proteome is ideal for proteomic discovery. The use of quantitative proteomics to explore differential spectral counts between CAD patients and healthy control subjects, facilitated the identification of a unique proteomic signature pathognomonic of CAD (Vaisar *et al*, 2010). A model comprising of the HDL associated proteins; apoA1, apo(a), apoC3 and apoC1, were found to be strong discriminators for CAD, when analysed collectively rather than individually. The diagnostic performance of the model was assessed using receiver operating characteristic (ROC) curves, which yielded an impressive area under the curve of 0.82 (Vaisar *et al*, 2010). This study emphasised the role of the HDL associated proteins in the diagnosis CAD.

5.2 **Aims**

For the purposes of this study Caucasian patients with CAD were compared to healthy Caucasian control subjects without CAD. The aim of this study was to identify novel proteins that may distinguish between the two populations and aid in the assessment of early detection of CAD, prior to symptom onset.

5.3 Materials and Methods

5.3.1 Materials and reagents

All materials and reagents that were used are described in Chapters 2 & 3.

5.3.2 Study Design

The lipoproteomic exploration was confined to a male population, as females have higher levels of HDL, due to endogenous and exogenous oestrogens which can alter the lipoproteome (Bradley *et al*, 1978).

It was evident that the statin therapy induced alterations in the protein cargo of the lipoproteins (Chapter 4). In any study, the comparison between controls and disease is subject to inherent confounding factors. The differences observed in the disease population may be a result of the drug therapies, rather than due to the actual pathobiology of the disease. Noticeably, in the study by Vaisar, blood sampling was performed 6 weeks after statin washout to eliminate any effect of drug therapy on the HDL proteome, in the comparison between CAD patients and controls (Vaisar *et al*, 2010). The withdrawal of statin therapy in a CAD group would be reviewed unfavourably by an ethical board for discovery purposes. In the current climate of increasing statin prescription, a more clinically relevant and valid approach, would be to match the cases and controls for statin therapy, to negate such effects on the lipoproteins. Furthermore, the approach by Vaisar was biased towards the HDL proteome, but the other lipoproteins also contain unique protein cargoes, albeit being smaller, it would therefore be prudent to explore all cargoes in an unbiased manner (Vaisar *et al*, 2010). Hence careful matching of the disease/control populations for age, gender, ethnicity and drug therapy was essential for plausible comparisons to be made and for accurate and congruent conclusion to be drawn from such results.

5.3.3 Patient Recruitment

Patients with known CAD were recruited into this prospective cohort study. This study complied with the declaration of Helsinki and was approved by the local ethic committee (REC reference: 13/EM/0049). Male Caucasian patients were recruited after a minimum of 3 months post ACS event and after a minimum of 1 month of statin stabilisation.

Healthy male Caucasians control subjects free from overt cardiovascular disease but who were on statin therapy were entered into this study. Written informed consent was sought from all patients. A questionnaire regarding the patient's medical history and medications was collated and confirmed with the medical notes. Blood pressure was recorded three times in resting patients using calibrated sphygmomanometers. Waist and hip measurements were recorded. Patient's height and weight measurements were recorded on a calibrated stadiometer. Patients with a known history of heart failure, ejection fraction below 45%, renal disease (eGFR below 50), inflammatory diseases or malignancy were excluded from this study. Modified diet in renal disease formula was used to calculate glomerular filtration rate (Stevens *et al*, 2007).

5.3.4 Angiographic analysis

The coronary angiograms for the patients were assessed by a cardiologist who was blinded to their risk factors and diagnosis. Significant CAD was defined as a coronary luminal stenosis greater than 50% in either a one vessel, two vessels or three vessels or the major branches of the coronary arteries.

5.3.5 Cardiac magnetic resonance imaging

To eliminate any possibility of subclinical CAD in the control cohort, cardiac magnetic resonance (CMR) imaging was performed to identify any regional wall motion abnormalities. Control subjects with regional wall motion abnormalities were excluded from this study.

5.3.6 Sample Preparation

The sample preparation is described in section 3.3.4.7. A pooled QC consisting of 10 µl from each CAD patient sample was created. The pooled CAD QC was used to ensure platform reliability and consistency. Furthermore, a pooled control QC consisting of 10 µL from each control sample was created. Samples were reconstituted in 0.1 % FA and spiked with an equal volume of 100 fmol of internal standard ADH (Waters Ltd.). Reconstituted samples were run in batches of 10. Prior to each respective batch, HeLa protein digest standard (Thermo Fisher Scientific Ltd) and either CAD or control QC's were injected on the LC-MS/MS (Waters Ltd.) to ensure optimal performance of the MS. A combination of HeLa standard, yielding over 2000 protein hits and QC protein hits within 10 % were deemed satisfactory for the running of the subsequent batch. Initially 1µl scouting injections for each sample were performed over a 75 minute gradient. Triplicate injections were performed for each sample over a 110 minute gradient in $HDMS^E$. Each sample injection aimed to load 500 ng onto the analytical column.

5.3.7 Statistical analysis

Statistical analysis was performed using SPSS version 22 (IBM, US). Continuous variables were analysed using the independent t-test. Categorical variables were analysed using Chi squared with continuity correction. In instances when continuity correction was not possible Fisher's exact test was used. The relationship between two continuous variables was assessed using the Spearman Rho correlations. Scatter graphs were constructed to display the relationship between two continuous variables. Boxplots were used to show the trend between continuous variables and discrete variables, with the line representing the median value and the whiskers of the boxplot representing the 25 th and 75 th centiles. In this study, linear regression analysis was unsuitable for the prediction of CAD status due to the limited number of observed cases relative to the predictor variables. Discriminant analysis was therefore performed to create a model that predicted CAD status using a group of uncorrelated variables. The baseline predictor variables that were entered into the model included age, history of diabetes, history of hypertension, smoking status, BMI, total cholesterol, HDL-C,

LDL-C, eGFR and the significant differentially expressed proteins. Continuous variables were expressed as mean ± SD, categorical variables expressed as percentages. A P value below 0.05 was deemed statistically significant.

5.4 Results

5.4.1 Clinical Demographics

The demographic and clinical data for the CAD patients (n=21) and the control subjects (n=7) is presented in Table 5.1. The CAD patients were compared with age, sex and race matched controls. There was no significant difference in prevalence of diabetes, hypertension or hypercholesterolemia between the disease and control groups. There was a higher proportion of ex-smokers in the CAD cohort compared with the control cohort, (81.0 % vs. 28.6 %; P=0.035) respectively. A higher proportion of the controls had never smoked compared to the CAD patients, but failed to reach statistical significance (57.1 % vs. 14.3 %, P=0.078). Of the CAD patients that had a history of MI, the majority were NSTEMI. The two groups had comparable haemodynamic parameters i.e. heart rate and blood pressure. The CAD patients had significantly higher BMI compared to the control group (29.76 kg/m^2 ± 5.25 vs 25.24 ± 1.92; P=0.037). The renal functions were similar between the two cohorts. Concerning the lipid parameters, there was no significant difference in total cholesterol or LDL levels between the control subjects and CAD patients, most likely to be attributable to the equivalence in statin prescription. However, the HDL levels were significantly higher in the control group compared to the CAD group (1.90 mmol/L ± 0.45 vs. 1.18 ± 0.26; P<0.001). Furthermore, the unfasted triglyceride levels were significantly higher in the CAD patients compared with control subjects (1.49 mmol/L ± 0.69 vs. 0.44 ± 0.42; P=0.001) respectively. Unsurprisingly, the prescription of cardiovascular medications; aspirin, β blocker, and ACEi/ARB were significantly higher in the CAD group compared to the control group. All patients analysed in this study were on statin therapy.

Table 5.1 Demographic and clinical data for the Control subjects and the CAD patients. Continuous variables are presented as means ± SD. Categorical variables are presented as percentages (%).

	Controls (n=7)	Caucasians CAD (n=21)	P value
Age	76±6.65	71±3.15	0.103
Gender (% male)	100	100	
Diabetes (%)	14.3	23.8	1.000
Hypertension (%)	57.1	42.9	0.827
Hypercholesterolemia (%)	85.7	61.9	0.483
Current smoker (%)	14.3	4.8	1.000
Former smoker (%)	28.6	81.0	0.035
Never smoked (%)	57.1	14.3	0.078
Previous MI (%)	0	85.7	<0.001
Previous CABG (%)	0	9.5	1.000
Previous PCI (%)	0	90.5	<0.001
Type of infarction			
STEMI (%)	0	43.8	
Physical Characteristics			
Heart rate (bpm)	64.38±6.93	59.19±9.76	0.207
SBP (mmHg)	147.33 ± 21.61	144.65±16.36	0.731
DBP (mmHg)	79.52±8.94	78.21±9.12	0.742
BMI (kg/m^2)	25.24 ± 1.92	29.76 ± 5.25	0.037
Biochemical data			
Urea	7.24±1.70	6.64± 1.13	0.295
Cr	82.57±13.20	86.81±17.21	0.558
eGFR_MDRD	86.36± 18.18	84.26± 21.48	0.819
Total cholesterol (mmol/L)	2.53 ± 2.40	3.66 ± 0.70	0.263
LDL (mmol/L)	1.26± 1.20	1.81 ± 0.55	0.279
HDL (mmol/L)	1.90 ± 0.45	1.18 ± 0.26	<0.001
Triglycerides (mmol/L)	0.44 ± 0.42	1.49 ± 0.69	0.001
Medications			
Aspirin (%)	14.3	85.7	0.002
B blocker (%)	0	81.0	0.001
ACEi/ARB (%)	28.6	85.7	0.016
Statin therapy (%)	100	100	

5.4.2 Protein identification

Raw data was analysed using PLGS software with 350_50_750 threshold for the high energy, low energy and intensity respectively. There was no significant difference in the number of protein hits between the CAD patients and the control patients (126.02 ± 8.84 vs. 124.33 ± 3.11; P=0.573) respectively, Figure 5.1. The overall CV in the proteins hits for both the CAD patients and the control subjects were 7.01 % and 2.50 % respectively, which were both below the acceptable threshold of 15%, indicating reliable and consistent data acquisition, Figure 5.2.

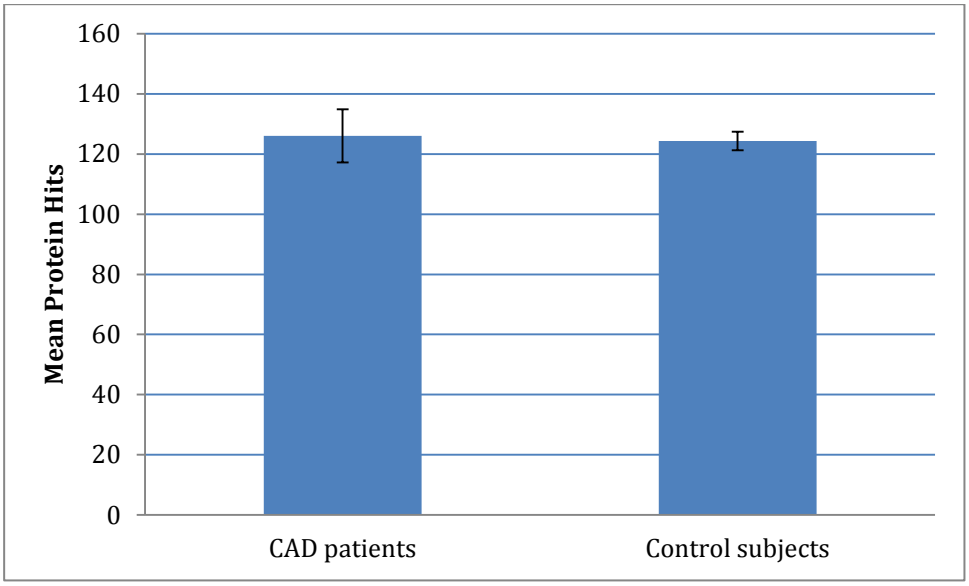


Figure 5.1 Bar chart showing the protein hits for the CAD patients and the Controls subjects. Bars represent the mean protein hits and the errors bars represent the SD.

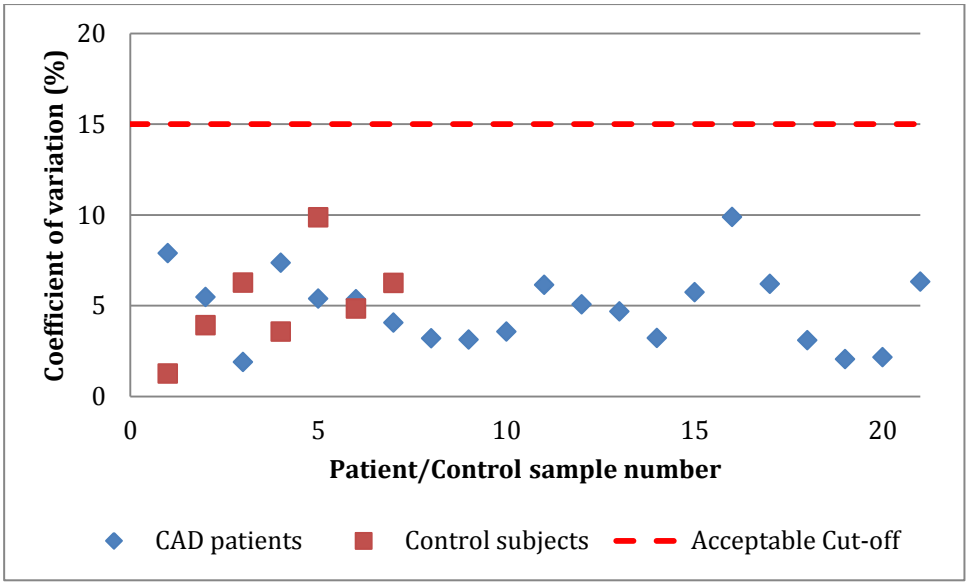


Figure 5.2 Coefficient of variation for the triplicate protein hits for each CAD/Control sample.

5.4.3 Protein Expression

Using the progenesis QI software for expression analysis, 272 proteins were identified in each group. One hundred and sixty eight proteins demonstrated significant differential expression between the CAD patients and the age, sex and ethnicity matched controls, Figure 5.3 & Figure 5.4. A selected number of proteins are presented in Table 5.2.

Table 5.2 Proteins demonstrating significant differential expression between the CAD patients and the age and sex matched controls.

Protein	Mean CAD protein intensities	Mean Control protein intensities	Fold change (Controls:CAD)	T Test
Extracellular matrix protein 1	29817.68 ± 7854.29	9001.69 ± 2274.01	3.31	3.959E-11
Alpha-1-acid glycoprotein 2	6828.37 ± 1456.60	3344.16 ± 350.17	2.04	2.492E-10
Alpha-2-HS-glycoprotein	342458.45 ± 61929.50	129363.96 ± 34649.76	2.65	6.500E-10
Inter-alpha-trypsin inhibitor heavy chain H4	107619.8 ± 28958.17	220556.50 ± 18458.32	0.48	1.394E-09
Lipopolysaccharide-binding protein	10211.00 ± 3128.70	3451.90 ± 739.21	2.96	1.865E-09
Apolipoprotein A-I	566562.31 ± 381443.00	1652827.31 ± 216035.30	0.342	1.751E-08
Transgelin-2	1069.87 ± 362.25	256.58 ± 163.65	4.17	3.325E-08
Insulin-like growth factor II	1809.43 ± 594.61	619.23 ± 237.31	2.92	7.067E-08
Mannan-binding lectin serine protease 1	78804.05 ± 26062.22	34091.01 ± 6391.20	2.31	1.35E-07
Serum amyloid A-1 protein	21528.20 ± 4577.87	12354.26 ± 1977.92	1.74	1.43E-07
Complement factor H-related protein 5	32143.89 ± 20504.52	1675.66 ± 1255.95	19.18	1.23E-06
Apolipoprotein B-100	296240.25 ± 103958.60	531850.14 ± 66385.50	0.56	2.680E-06
Tubby-related protein 2	5283.38 ± 1372.02	2645.27 ± 791.82	2.00	6.261E-06
Hepatocyte growth factor-like protein	3432.12 ± 850.84	6531.43 ± 866.50	0.53	8.271E-06
Serum paraoxonase/arylesterase 1	70145.88 ± 18769.36	116371.39 ± 15041.62	0.60	1.853E-05
Vitronectin	56785.15 ± 29701.53	175476.91 ± 35860.32	0.32	2.580E-05
Apolipoprotein F	11365.64 ± 3555.23	6973.27 ± 1003.35	1.63	2.735E-05
Angiotensinogen	13478.30 ± 2140.48	44916.56 ± 8195.70	0.30	4.279E-05
GXP 3	4494.76 ±	22794.79 ±	0.20	2.040E

	2373.28	6394.02		-04
Galectin-3-binding protein OS=Homo sapiens GN=LGALS3BP PE=1 SV=1	27677.05 ± 15723.61	12035.71 ± 2222.04	2.30	2.082E -04
Carboxypeptidase B2	34248.30 ± 10670.05	85363.97 ± 19952.73	0.40	3.072E -04
Isoform 2 of Clusterin	330015.12 ± 105289.40	568693.78 ± 116402.60	0.58	8.240E -04
Prothrombin	112920.23 ± 52072.13	67080.89 ± 14315.25	1.68	0.001
Complement factor D	18717.67 ± 5330.03	30482.21 ± 6522.30	0.61	0.002
Peroxiredoxin-2	354.20 ± 278.07	137.42 ± 48.11	2.58	0.002
Alpha-1-acid glycoprotein 1	79.93 ± 128.57	1214.78 ± 798.00	0.07	0.009
Apolipoprotein C-II	36411.97 ± 11334.73	23835.06 ± 9419.21	1.53	0.013
Apolipoprotein(a)	10091.52 ± 3536.96	7910.43 ± 672.97	1.28	0.013
Cartilage acidic protein 1	673.07 ± 264.06	1013.87 ± 264.32	0.66	0.014
Thrombospondin-1	794.23 ± 434.04	1548.71 ± 615.90	0.51	0.017
Alpha-1-antichymotrypsin	36273.95 ± 11452.60	29740.67 ± 1285.55	1.22	0.018
Complement C3	463962.40 ± 132199.30	561294.80 ± 80812.11	0.83	0.033

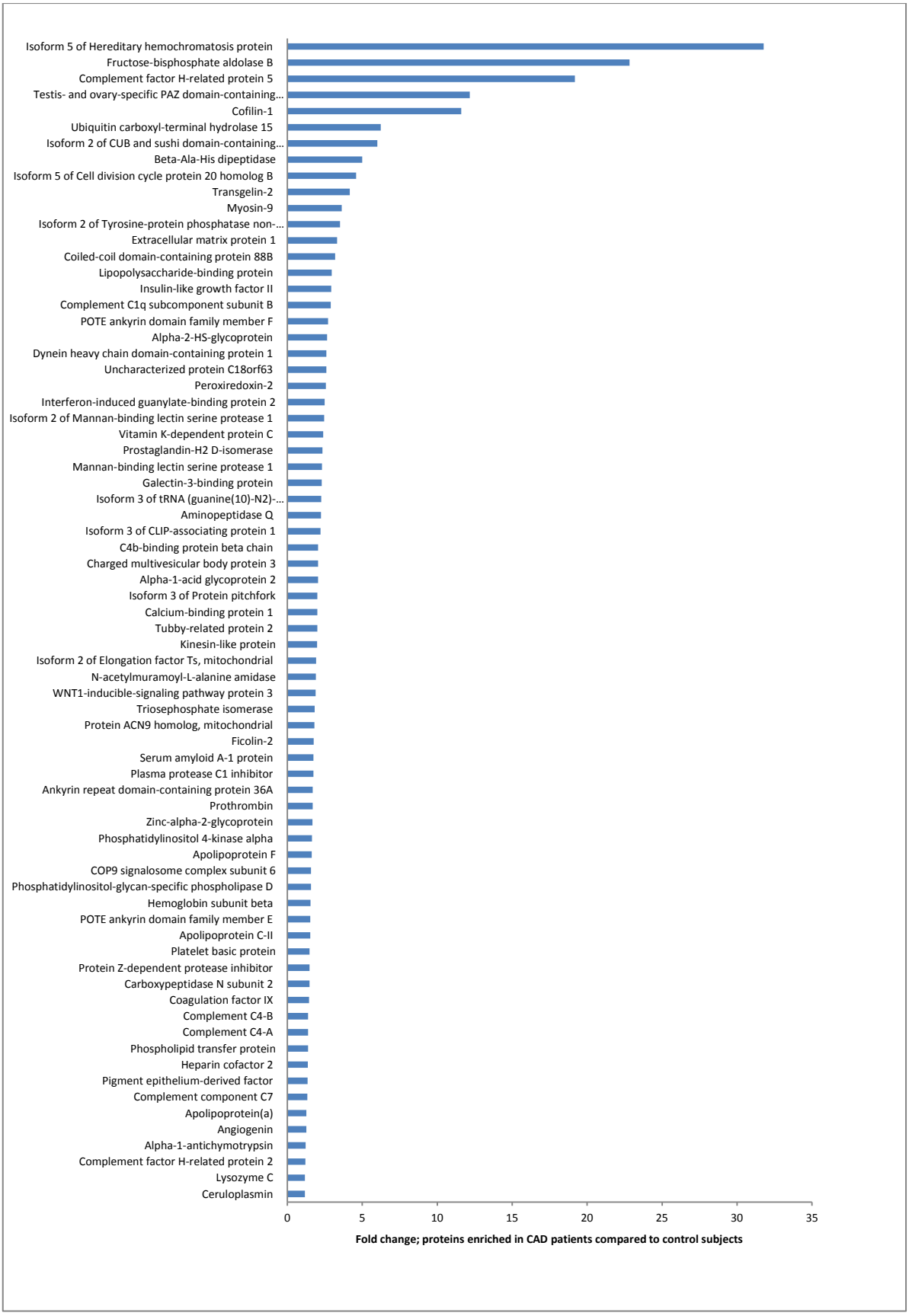


Figure 5.3 Significant proteins over expressed in the CAD patients compared to the control subjects (Fold change>1). Data derived for triplicate analysis in Progenesis QI for Proteomics. Data subject to t-test analysis.

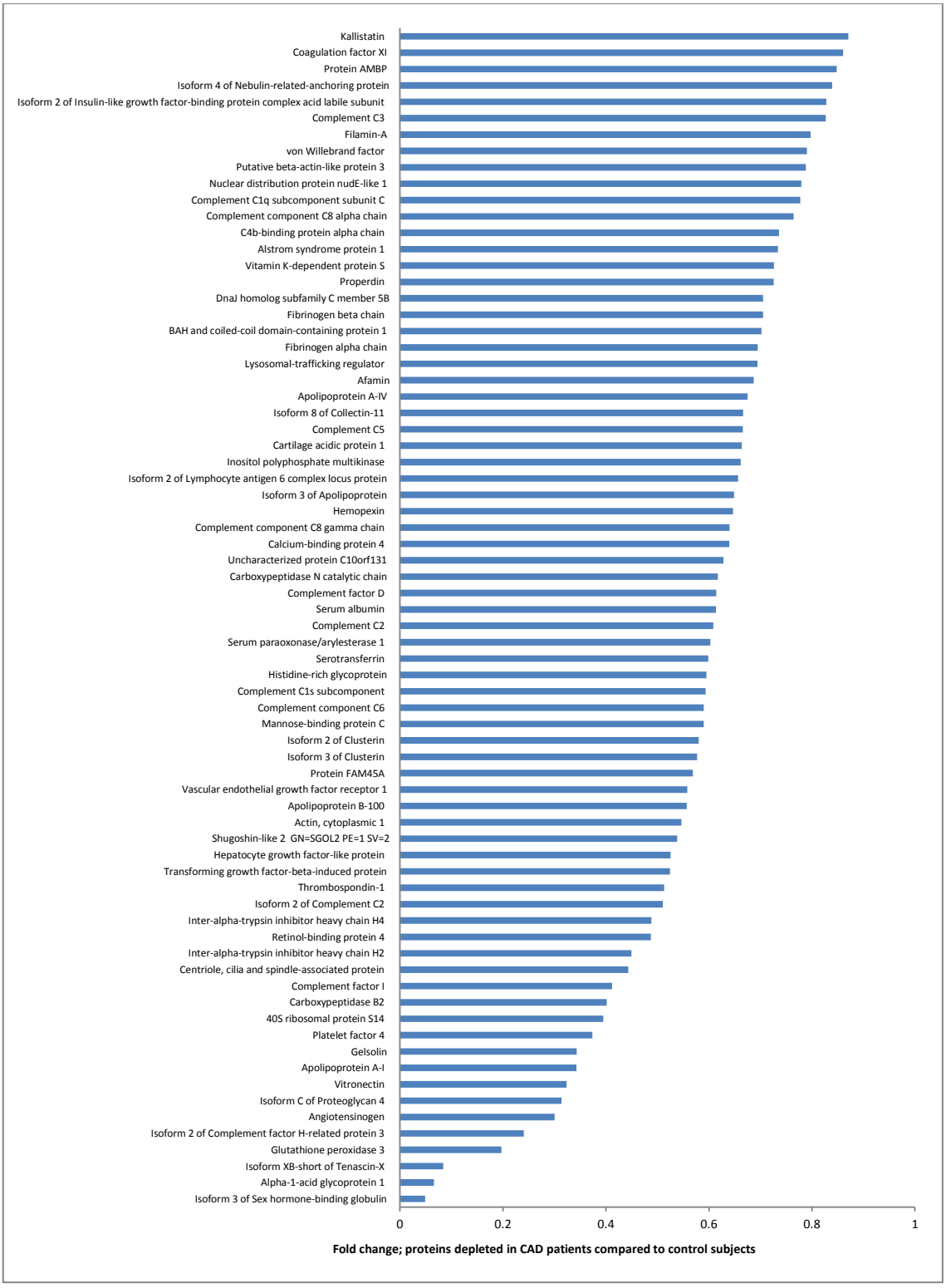


Figure 5.4 Significant proteins depleted in the CAD patients compared to the control subjects (Fold change <1). Data derived for triplicate analysis in Progenesis QI for Proteomics. Data subject to t-test analysis.

5.4.4 Correlations

To assess the association between the laboratory determined HDL and LDL levels and their respective constituent apolipoproteins, Spearman rho correlations were performed. There was a significant linear relationship between HDL-C and apoA1 intensity levels (rs=0.544, P=0.005) Figure 5.5. There was no relationship between LDL-C and apoB100 levels (rs=-0.187, P=0.341) Figure 5.6. The lack of an association between the LDL-C and the apoB100 intensity, may be because the apoB100 intensity is not simply reflective of a single lipoprotein, but of all the apoB100 containing lipoproteins i.e. VLDL. Therefore, the increased apoB100 signal may diminish any possible association with LDL-C, explaining the inverse relationship.

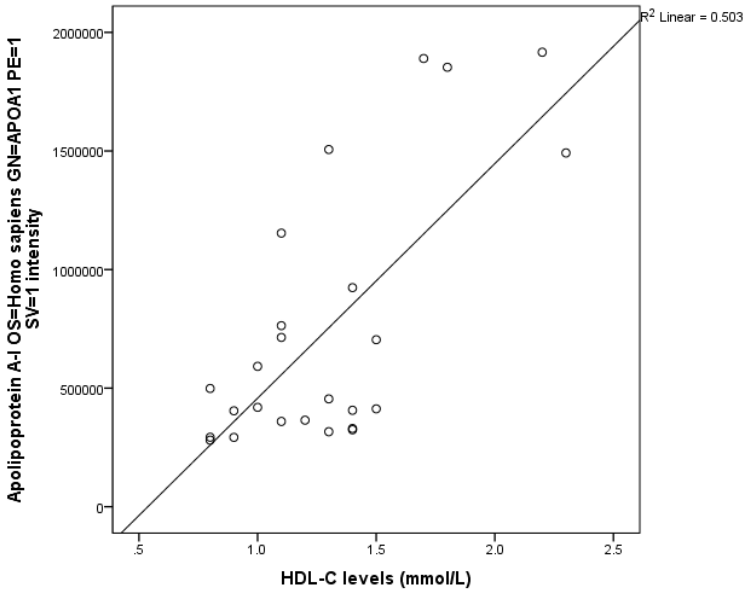


Figure 5.5 Scatter graph showing the relationship between HDL-C levels and apoA1 intensity (rs=0.544, P=0.005).

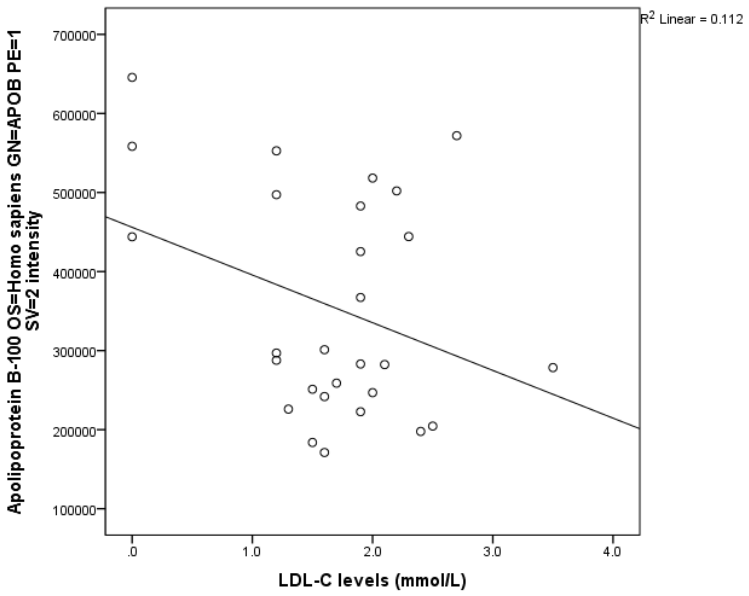


Figure 5.6 Scatter graph showing the relationship between LDL-C levels and ApoB100 intensities (rs=-0.187, P=0.341).

The apoA1 intensities were significantly lower in the CAD cohort compared with the age and sex matched controls (mean ± SD, 566562.21 ± 381443.05 vs. 1652827.31 ± 216035.28; P=1.75e^{-10}) respectively, which is consistent with the HDL-C observations Figure 5.7. In contrast, apoB100 levels were significantly lower in the disease group compared with the control group (mean ± SD, 296240.25 ± 103958.60 vs. 531850.14 ± 381443.05; P=2.68e^{-6}) respectively, Figure 5.8. The lower apoB100 levels in the disease cohort are likely to be a consequence of the higher intensity statin dosing in the CAD group, exerting a greater effect on the apoB100 containing lipoproteins compared to the controls.

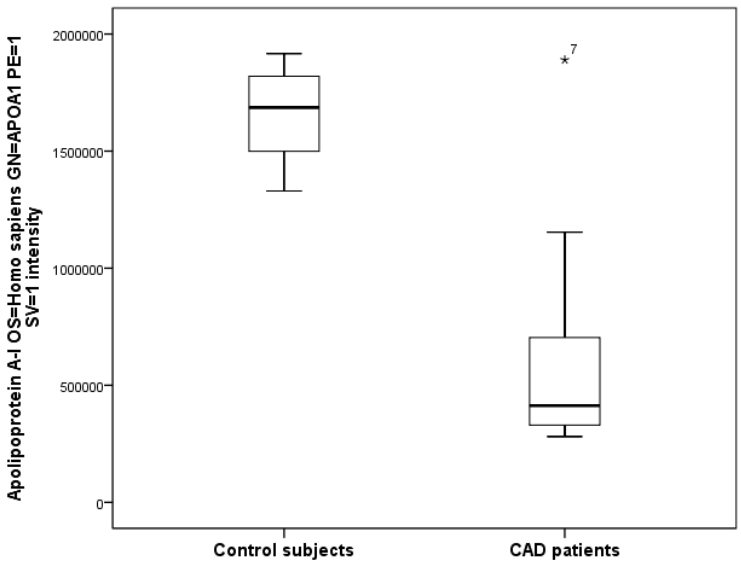


Figure 5.7 Box plots showing the apoA1 intensity between the control subjects and the CAD patients (mean ± SD, 1652827.31 ± 216035.28 vs. 566562.21 ± 381443.05 respectively; $P=1.75e^{-10}$).

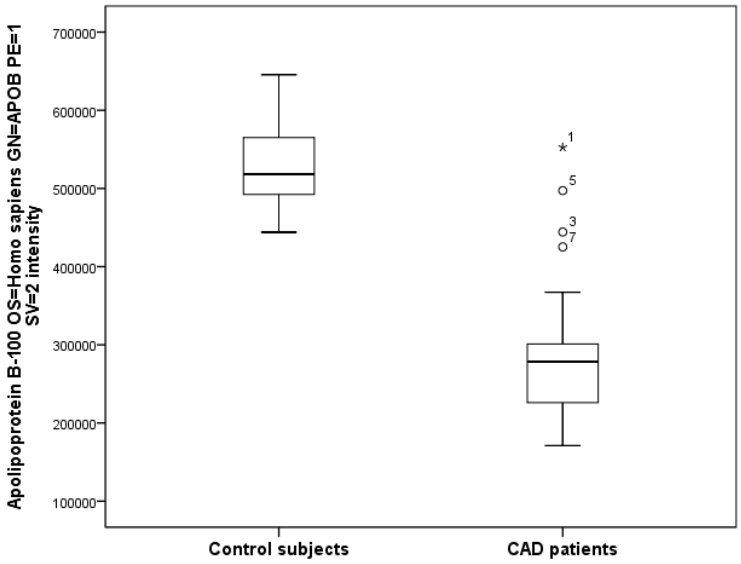


Figure 5.8 Box plot showing the apoB100 intensities between the control subjects and the CAD patients (mean ± SD, 531850.14 ± 381443.05 vs 296240.25 ± 103958.60 respectively; $P=2.68e^{-6}$).

5.4.5 Discriminant analysis

To determine the independent predictors for the occurrence of CAD status, discriminant analysis was used in preference to logistic regression due to the limited sample set. Discriminant analysis identifies a group of variables that exert the strongest effect on the dependent variables which can therefore accurately predict group/outcome membership. In discriminant analysis the dimensionality of the data is condensed to identify linear relationships with the dependent variable.

In this analysis, variables known to influence the occurrence of CAD were entered into this stepwise discriminant analysis. In addition, the variables that were significantly related to CAD status were also entered. Seven discriminating variables were identified that were significantly predictive of CAD status ($P<0.001$). The model was impressive in explaining all the variation in the outcome. From Table 5.3, it is evident that carboxypeptidase B2 exerted the largest standardised canonical discriminant function coefficient, indicating it has the largest effect on CAD status, compared to the other predictor variables. In classification analysis the predictor model was able to correctly assign each case to the correct group. All patients with CAD and those without CAD were correctly classified, indicating strong predictive utility of this model, Table 5.4. Furthermore, in cross validation analysis, all subsequent cases were correctly classified, confirming the efficacy of this model for the prediction of CAD.

Table 5.3 Standardised canonical discriminant function coefficients for the significant proteins in the final discriminant analysis model.

Standardised Canonical Discriminant Function Coefficients	
	Function 1
ACT	-1.643
Angiotensinogen	3.321
Apolipoprotein CII	1.111
Carboxypeptidase B2	4.158
Complement C3	-3.575
ITIH4	2.595
Isoform5 of Hereditary hemochromatosis protein	-3.619

Table 5.4 Classification results for the prediction of CAD status using the discriminant predictor model.

Classification Results[a,c]					
		CAD=1, Control= 0	Predicted Group Membership		Total
			0	1.0	
Original	Count	0	7	0	7
		1.0	0	21	21
	%	0	100.0	0	100.0
		1.0	0	100.0	100.0
Cross-validated[b]	Count	0	7	0	7
		1.0	0	21	21
	%	0	100.0	0	100.0
		1.0	0	100.0	100.0
a. 100% of original grouped cases correctly classified.					
b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.					
c. 100% of cross-validated grouped cases correctly classified.					

5.4.6 Pathway Analysis

The differentially expressed proteins between the CAD patients and the controls subjects were entered into STRING software, to establish protein interactions, Figure 5.9 (Szklarczyk *et al*, 2015). Clustering analysis revealed 4 main functional protein clusters, Figure 5.10. The main functional clusters centred on apoA1, albumin, complement C3 and complement C8-alpha.

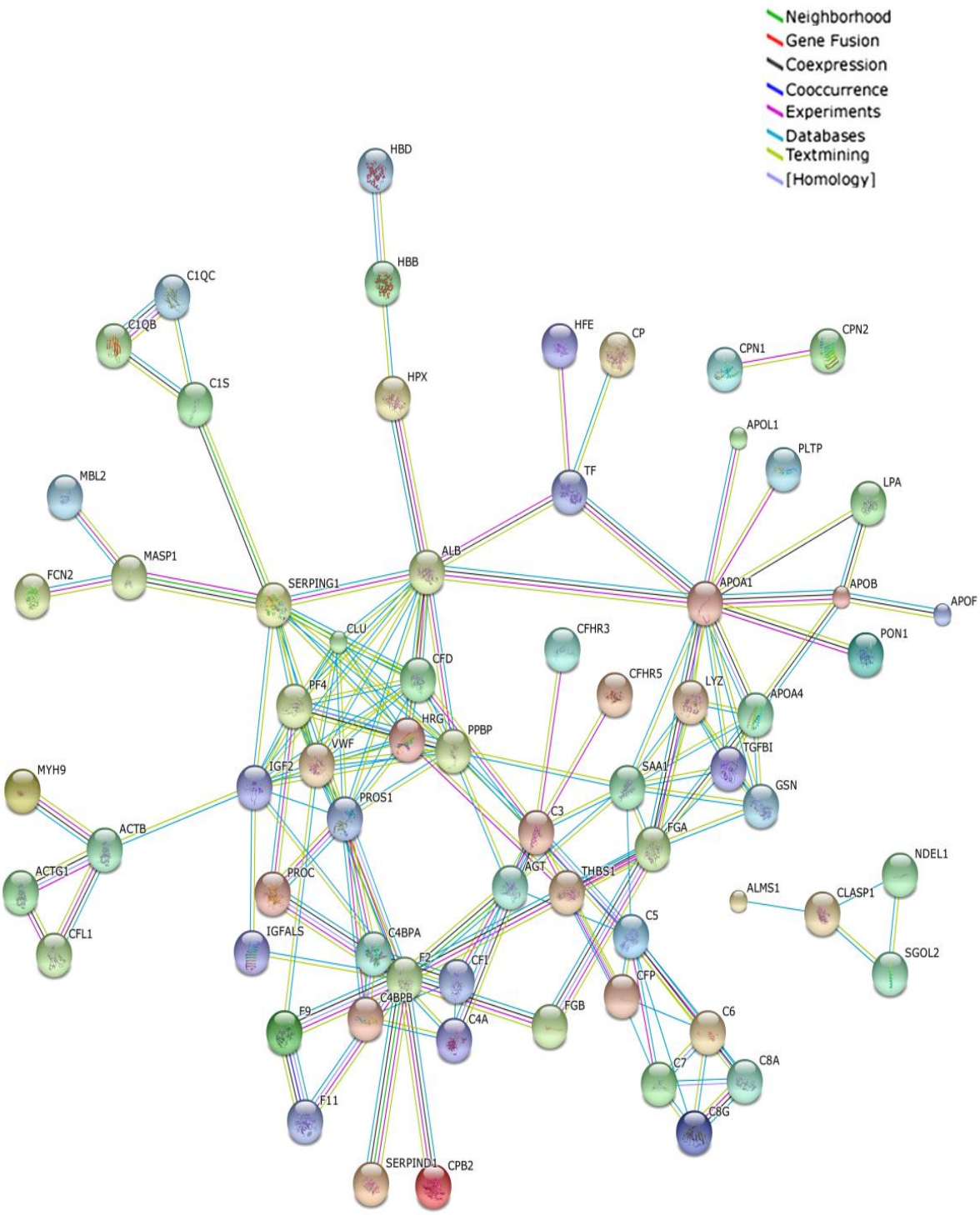


Figure 5.9 Protein-protein interaction networks visualised by STRING. Protein-protein interaction networks of the dysregulated proteins in the CAD patients compared to the control subjects. The different colours represent the different levels of evidence for the interactions.

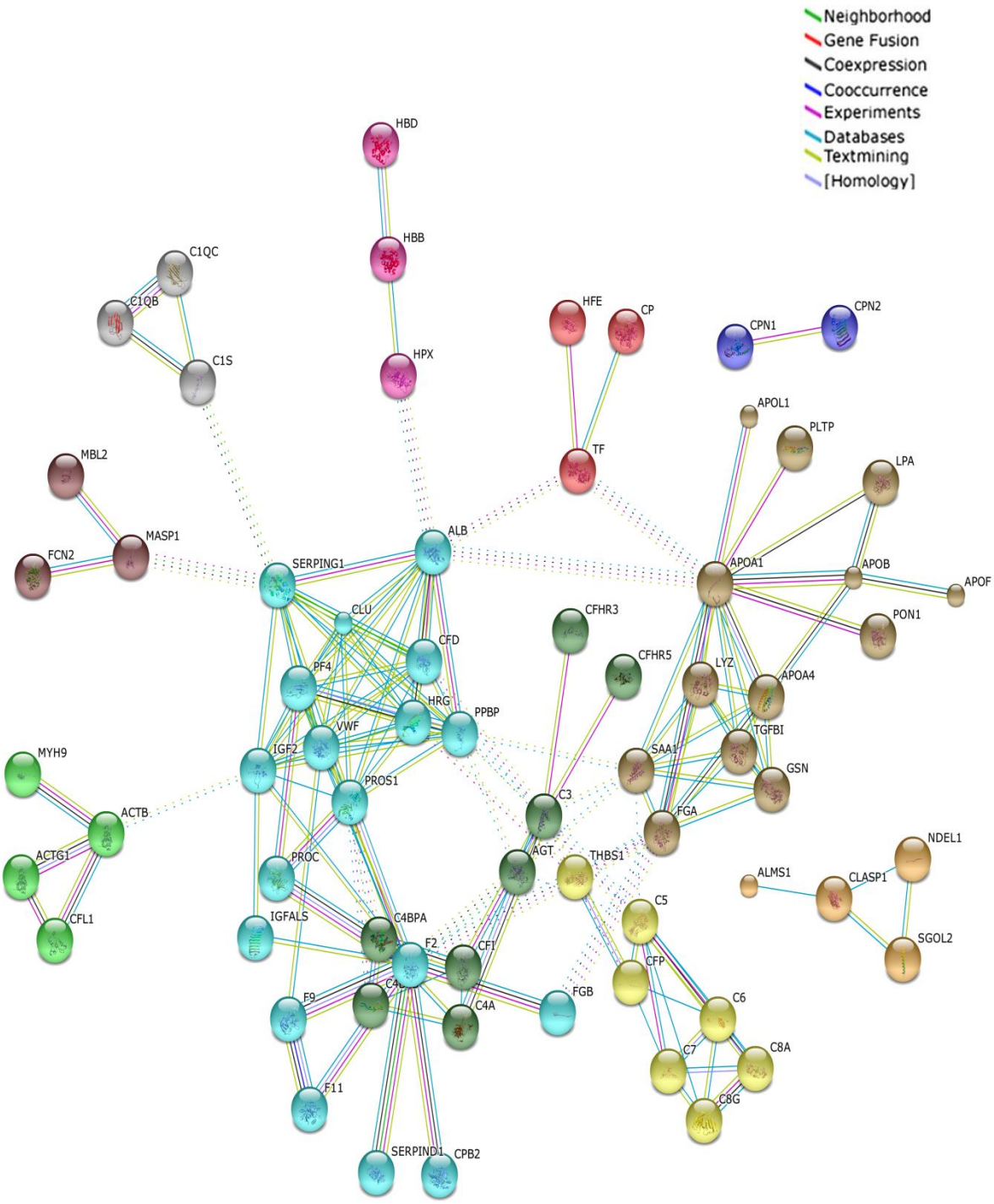


Figure 5.10 Clustering of the protein interaction networks visualised by STRING. Protein-protein interaction networks of the dysregulated proteins in the CAD patients compared to the control subjects. The different coloured nodes represent the different protein clusters. The coloured lines represent the different levels of evidence for the interactions.

5.5 Discussion

5.5.1 Complement C3

In this study complement C3 levels were significantly lower in the CAD patients compared with the age and sex matched controls (mean ± SD, 463962.42 ± 132199.33 vs. 561294.84 ± 80812.11; P=0.033). Furthermore, in discriminant analysis it was selected as a significant discriminator between disease cases and control cases. Complement C3 is a key component of the complement system, with all three pathways (classical, alternative, lectin) converging to activate this protein to mediate an inflammatory response. In murine models of atherosclerosis, LDL/C3 null mice had greater atherosclerotic lesions with increased macrophage content compared with control mice (Buono *et al*, 2002). It was suggested that C3 may have a modulating effect on the progression of fatty plaques to more complex lesions, as the LDL/C3 null mice had reduced collagen and smooth muscle content in the plaques compared with control mice (Buono *et al*, 2002). In another murine model of atherosclerosis, the triple knockout for LDL, apoE and C3 resulted in larger atherosclerotic lesion combined with an atherogenic lipid profile compared with control mice, suggesting that the complement system may be protective (Persson *et al*, 2004). By contrast, in humans, raised levels of C3 and C4 were associated with an increased incidence of CAD (Engstrom *et al*, 2007). The association for C3 disappeared after correcting for the conventional risk factors, but remained for C4, with patients in the 90th centile observing the highest risk of adverse events compared to those in those in the lowest centile (Engstrom *et al*, 2007). The increased C4 levels would support complement activation and contribute to the microcosm of inflammation that underpins atherosclerosis. Interestingly, the HDL particle undergoes inflammatory change in ACS with the predominance of inflammatory mediators. It was shown in a study by Alwaili, that the HDL particle becomes enriched with C3 in ACS patients compared with control subjects (Alwaili *et al*, 2012). However, no significant enrichment/depletion in C3 levels was observed between the stable CAD patients and the control subjects.

The role of medication, in particular, statins cannot be overlooked as a potential confounder. A study by Mason et al demonstrated that statins regulate the complement system by increasing the expression of membrane bound decay

accelerating factor (DAF) which inhibits the formation of C3 and C5 convertase, thereby reducing C3 levels (Mason *et al*, 2002; Fujita *et al*, 1987). It was revealed that both simvastatin and atorvastatin increased both mRNA transcripts for DAF and the actual protein levels in HUVEC in a dose dependent manner (Mason *et al*, 2002). Furthermore, atorvastatin reduced cellular expression of C3 by up to 60%, protecting the cell from lysis (Mason *et al*, 2002).

In this current study, all control subjects (n=7) were taking statin therapy to minimise any effects on the lipoprotein proteomes, however in the study by Alwaili, only one control subject was taking statin therapy (n=10) (Alwaili *et al*, 2012). In this current study, the majority of the stable CAD patients (66.7 %) were on doses higher than 20mg. It is therefore plausible that the higher doses of statins in the CAD group may exert a greater suppressive effect on C3 levels, probably via increased DAF levels, compared with the control cohort.

5.5.2 Apolipoprotein C2

In this study apoC2 intensity levels were significantly higher in the CAD patients compared with the age and sex matched controls (mean ± SD, 36411.97 ± 11334.73 vs. 23835.06 ± 9419.21, P=0.014) respectively. Furthermore, it was identified as a significant predictor of CAD status in discriminant analysis. ApoC2 belongs to the apoC family which are primarily concerned with triglyceride metabolism. ApoC is synthesised in the liver and incorporated in chylomicrons and VLDL particles. It is primarily concerned with the activation of lipoprotein lipase, which hydrolyses the triglycerides to free fatty acid. Elevated levels of apoC2 would theoretically support increased triglyceride metabolism. Counter-intuitively, the over expression of apoC2 in mice resulted in increased triglyceride levels in both the fasting and non-fasting state (Shachter *et al*, 1994). Furthermore, the higher triglyceride levels favoured the formation of larger VLDL particles. It was inferred that higher apoC2 expression promoted the hypertriglyceridaemia through reduced interaction with the lipoprotein lipases and clearance receptors (Shachter *et al*, 1994).

In contrast, a HDL proteomic analysis revealed depleted apoC2 levels in CAD patients compared to healthy control subjects (Riwanto *et al*, 2013). This discrepancy may be a treatment effect, as the control group were drug naive. However, in chapter 4, apoC2 levels did not to change with statin therapy (P=0.100). An alternative explanation is that the apoC2 levels in this current study are not just solely reflective of the HDL cargo, but also of the entire apoB containing lipoprotein cargoes. The affinity resin was not biased to a particular lipoprotein class, so the apoC2 levels are likely to be reflective of the total lipoprotein content, rather than a particular class. Furthermore, as with all apolipoprotein except for apoB100, their ability to exchange between different classes may be relevant and is often overlooked, hence the use of an unbiased affinity resin overcomes this issue.

In this current study apoC2 levels were highly correlated with triglyceride levels (rs=0.549, p=0.002), which is consistent with that observed by Shachter in the transgenic mouse model of apoC2 (Shachter *et al*, 1994). No significant correlations were observed between the other apoC family members and the triglyceride levels. In addition, apoC2 intensities were positively correlated with apoC1 (rs=0512, P=0.005), apoC3 (rs=0.529, P=0.004) and apoC4 (rs=0.549, P=0.002). The direct relationship between apoC2 and apoC3, suggests that apoC2 may act in a similar manner to apoC3 contributing to hypertriglyceridaemia.

The role of hypertriglyceridaemia in CAD is of growing importance, as a recent study showed a strong link with high triglycerides levels and adverse risk after ACS in patients from the Dal-outcomes and MICRL studies, despite treatment with statins (Schwartz *et al*, 2015). It was revealed that patients in the highest quintile for triglycerides had a 1.5-fold increased risk of adverse events compared to those in the lowest quintile (Schwartz *et al*, 2015).

5.5.3 Inter alpha trypsin inhibitor heavy chainH4

In this study inter alpha trypsin inhibitor heavy chain H4 (ITIH4) levels were significantly lower in the CAD patients compared with the control subjects (mean ± SD, 107619.82 ± 28958.17 vs. 220556.45± 18458.32; P=1.394e^{-9}) respectively.

This protein was recognised as a significant discriminator between CAD and control subjects. ITIH4 is an acute phase protein that is synthesised by the liver. A recent study by Xu et al reported that lower levels of ITIH4 were observed in stable CAD patients that endured adverse events at one year, compared to event free survivors (Xu *et al*, 2013). At the other end of the cardiovascular spectrum, acute ischaemic stroke was associated with complete absence of ITIH4 compared with controls subjects, with levels eventually normalising with clinical improvement (Kashyap *et al*, 2009).

ITIH may have a role in cholesterol metabolism, as a SNP in the ITIH4 gene, was associated with higher total cholesterol levels in a Japanese cohort (Fujita *et al*, 2004). Patients, who lacked the T allele in the SNP at a splice site in the ITIH4 gene, had significantly higher total cholesterol levels and a higher prevalence of hypercholesterolemia compared to those with the T allele (Fujita *et al*, 2004). This genomic data may reinforce proteomic findings, as this protein is known to reside within the HDL proteome and therefore may have a role in the cholesterol pathway (Vaisar *et al*, 2007). However, in this current study no significant direct correlations were observed between the ITIH4 intensity levels and total cholesterol, HDL or LDL levels.

5.5.4 Alpha-1-anti-chymotrypsin

In this study, alpha-1-anti-chymotrypsin (ACT) also known as serpin A3, was significantly higher in the CAD patients compared to the control subjects (mean ± SD, 36273.95 ± 11452.60 vs. 29740.67 ± 1285.55; P=0.017). ACT was identified as a significant predictor of CAD status in discriminant analysis. ACT belongs to the SERPIN family and is concerned with the deactivation of serine proteases such as chymases and cathespin G (Horvath *et al*, 2005). It has also been implicated in the process of extracellular remodelling, as levels of ACT were elevated in carotid atherosclerotic lesion of symptomatic patients compared to lesions in asymptomatic patients (Wagsater *et al*, 2012). This finding was corroborated by increased expression of ACT mRNA in the atherosclerotic lesions. The expression of atherosclerotic ACT was postulated to be locally mediated rather than via the blood stream, as there was no significant difference in plasma samples between

ACS patients and controls (Wagsater *et al*, 2012). The lack of differential plasma expression between the ACS patients and controls is surprising, considering ACT has been referred to as an acute phase protein. In contrast, work by Riwanto, revealed that the HDL of CAD patients was significantly enriched with ACT compared to control subjects, which is consistent with the findings in this current study (Riwanto *et al*, 2013). The disparity in ACT expression between the current study and that of Wagstar, may simply be related to the higher sensitivity conferred by analytical mass spectrometry compared to immunoassays (Wagsater *et al*, 2012).

The elevated levels of ACT in the CAD patients may be protective. The inhibitory effects on chymases and cathespin may limit the pathological remodelling that occurs within the plaques, as evidenced by chymase inhibition which has shown to reduce plaque size and the necrotic core in apoE null mice (Bot *et al*, 2011).

5.5.5 Carboxypeptidase B2

In this study carboyxpeptidase B2 levels were significantly lower in the CAD patients compared to the controls (mean ± SD, 34248.30 ± 10670.05 vs. 85363.97 ± 19952.73, $P=3.07e^{-4}$) respectively. This protein was also identified as a significant discriminator of CAD status. Carboxypeptidase B2 (thrombin activatable fibrinolysis inhibitor) is synthesized by the liver and is activated by thrombin. Upon activation, it cleaves the C-terminal of the fibrin particle, which inhibits the binding of plasminogen to the fibrin surface and prevents its interaction with tissue plasminogen activator, ultimately impairing fibrinolysis (Bouma *et al*, 2001). There is considerable conflict in the literature as to which direction this protein confers protection or risk.

5.5.6 Angiotensinogen

In this study angiotensinogen levels were significantly lower in the CAD cohort compared with controls (mean ± SD, 13478.30 ± 2140.48 vs. 44916.56 ± 8195.70; P=4.279E-05) respectively. This protein is in integral in the renin angiotensin-aldosterone system and contributes to the downstream synthesis of angiotensin2

which is important in adverse cardiac remodelling and blood pressure control. This system has been the subject of intense pharmaceutical manipulation, with blockade of the angiotensin converting enzyme (ACE) and the angiotensin receptor, which have been shown to confer huge survival benefit post MI (Pfeffer *et al*, 1992; Kober *et al*, 1995). It is therefore feasible that blockade of this system is likely to result in lower angiotensinogen levels. This finding was also observed by Riwanto (Riwanto *et al*, 2013).

5.6 **Conclusion**

The advent of tools such as the Framingham risk score have assisted in the identification of high risk individuals for CAD (Kannel, McGee and Gordon, 1976). The incorporation of new markers has improved risk scoring accuracy; however, the majority of individuals with latent CAD are undiagnosed. The lipoproteins carry a diverse array of low abundant proteins that impart protective and deleterious functions. Hence the use of a lipoproteomic approach was cultivated to identify novel markers that may be used as predictors or discriminators of CAD. In this study, quantitative analytical mass spectrometry was used, to assess the lipoproteomic differences between stable CAD patients and age and sex matched healthy control subjects. Two hundred and seventy two proteins were identified between the two cohorts, with 168 demonstrating dysregulated expression. Pathway analysis revealed that the dysregulated proteins had diverse role in complement activation, redox reactions, inflammation, angiogenesis, coagulation and lipid metabolism. The dynamic array of properties reinforces the notion that the lipoproteins are not only transporters of lipid but are heavily intertwined in the pathogenesis of atherosclerosis (Vaisar *et al*, 2007). This is consistent with findings reported by Vaisar and Alwaili, that the proteomic cargo of the lipoproteins undergoes pro-inflammatory change (Alwaili *et al*, 2012; Vaisar *et al*, 2010). The appreciation of such a shift in the proteomic cargo may therefore give subtle clues to disease onset even before plaques have formed or matured. In stepwise discriminant analysis 7 proteins were identified as the strongest predictors of CAD status. Each protein imparted some effect on the outcome, but in

combination they were able to accurately classify the CAD patients and the control patients. The final model is presented below as potential markers of CAD in the future.

Table 5.5. The model provides multi-level information with an almost 3-dimensional perspective of the disease. ACT levels were higher in the CAD patients and most likely contribute to extracellular remodelling via the inhibition of the chymases.

Similarly, apoC2 levels were higher in the CAD cohort compared with the controls. Its direct relationship with triglyceride levels is consistent with its role in triglyceride metabolism.

ITIH4, an acute phase protein was found to be depressed in the CAD group, consistent with lower levels observed in acute ischaemic stroke and in patients with adverse outcome in CAD (Xu *et al*, 2013; Kashyap *et al*, 2009).

Carboxypeptidase B2 has an integral role in fibrinolysis, with reduced expression in the CAD cohort (Bouma *et al*, 2001; Buono *et al*, 2002). The observation of lower carboxypeptidase B2 levels is likely to represent a treatment effect from the anti-platelet medication.

Counter-intuitively, complement C3 levels were lower in the CAD group, which is consistent with a murine model, in which C3 knockout resulted in larger atherosclerotic lesions compared with control mice (Buono *et al*, 2002). However, this may also represent a treatment effect of statins, as they have been shown to reduce C3 formation by indirectly reducing C3 convertase (Mason *et al*, 2002; Fujita *et al*, 1987).

In addition, the lower levels of angiotensinogen in the CAD group, has been previously documented and may also represent a treatment effect.

A limitation of this study is the inability to properly match the disease and controls for medications. A prerequisite for entry into this study was for subjects to be on statin therapy, but it is obvious that other medications may be involved in altering the lipoproteome.

This model represents discovery work and needs to be replicated in a larger cohort of patients, to truly assess its diagnostic potential. Even with limited number of samples, differential protein expression was observed, emphasising that the lipoproteins are hugely dynamic and present a fantastic platform in which to identify novel markers of CAD in the future.

Table 5.5 Significant discriminators of CAD

Model	
1	ACT
2	Angiotensinogen
3	Apolipoprotein CII
4	Carboxypeptidase B2
5	Complement C3
6	ITIH4
7	Isoform5 of Hereditary hemochromatosis protein

Chapter 6

6 The impact of ethnicity on lipoproteins in CAD

6.1 Introduction

The South Asian population community is a heterogeneous group with varying behavioural and cultural beliefs. Within this group there is great variability in terms of diet, religious practices, health beliefs and social status. The environmental factors integrate alongside genetic factors to predispose this group to a higher incidence of CAD than their Caucasian counterparts.

The most pertinent variables contributing to the heightened risk of CAD in the South Asians community includes dysglycaemia eventually causing overt diabetes mellitus, abdominal obesity and dyslipidaemia. The prevalence of diabetes is higher in the South Asian community compared with the Caucasian community and represents the tip of an iceberg (Mostafa *et al*, 2010). It was revealed that the detection of diabetes amongst the South Asian community may be increased by the use of glycated haemoglobin compared to the conventional oral glucose tolerance test (Mostafa *et al*, 2010). Impaired fasting glucose is a prelude to the development of diabetes and is more common in the South Asian community. A study by Admiraal showed that the impaired fasting glucose as defined by fasting plasma glucose of 5.7-6.9 mmol/L was strongly associated with the development of diabetes 10 years later (Admiraal *et al*, 2014). The incidence was nearly 20 % for the South Asians compared to 4.5 % for the other Dutch ethnicities. Admiraal et al reported that the presence of impaired fasting glucose in the South Asians had a more toxic effect in translating to overt diabetes compared to the lower incidence of diabetes in the other minority ethnic individuals (Admiraal *et al*, 2014). The role of diabetes as a key driver for the development and progression of CAD is unquestionable. The higher incidence of diabetes in the South Asian community may be the most important aetiological factor for their development and progression of CAD.

Obesity is a global issue, with greater health care initiatives aimed at tackling this, to reduce the burden of cardiovascular disease in the future. The adipose tissue is a rich source of chemical signals called adipokines that communicate with various

organs, in particular the pancreas, regulating insulin secretion. The distribution of adipose tissue may be more vital in understanding the role of obesity in dyslipidaemia and dysglycaemia. The pattern of deep subcutaneous, intraperitoneal fat and visceral fat are associated with mediating such disturbances (Abate *et al*, 1995). A study by Raji et al. investigated the fat distribution in healthy South Asian Indians and healthy Caucasians (Raji *et al*, 2001). Interestingly, despite comparable BMI's between the two groups, the Asian Indians had more total abdominal fat and visceral fat compared with the Caucasians (Raji *et al*, 2001). In addition, fasting insulin levels were higher in the South Asian Indians compared to the Caucasians. The South Asian Indians had lower HDL and higher LDL, which were significantly correlated with visceral fat (Raji *et al*, 2001). It was recently hypothesised that the reason for the susceptibility towards abdominal obesity in the South Asians compared to the Caucasian, was that the reservoir of subcutaneous fat, the primary energy storage unit, was smaller in the South Asians, such that this storage unit would become exhausted earlier in the South Asians, with over spilling into the deeper adipose tissue and consequently contributing to their body habitus (Sniderman *et al*, 2007). The deeper abdominal and visceral adipose tissues were more harmful due to their rich vascularity enabling the transport of fatty acids into the blood stream causing dyslipidaemia (Sniderman *et al*, 2007). Although, BMI may be comparable between the two ethnicities the body fat distribution may be different, which has led to the acceptance of lower BMI thresholds for South Asians and the introduction and incorporation of waist hip ratio as a surrogate for abdominal obesity (Kumar *et al*, 2011).

Dyslipidaemia may be the final output from a culmination of various risk factors, which leads to the development of a fatty plaque through to the complicated complex atherosclerotic lesion. South Asians tend to have comparable total cholesterol levels compared with Europeans (Chambers *et al*, 2001). The focus of total cholesterol in the South Asian groups may be confounded by their lower HDL levels. In the South Asians, there is a predominance of the smaller denser HDL subspecies which are more atherogenic as previously described in 1.2.3.1(Bhalodkar *et al*, 2004). LDL levels in South Asians are comparable with

Caucasians, with the predominance of the atherogenic pattern B (Bhalodkar *et al*, 2004).

Culmination of these risk factors contributes to a more aggressive CAD in the South Asians community than the Caucasian community. A study by Koulaouzidis reported that South Asian patients with symptomatic angina had a greater burden of CAD than symptomatic Caucasians patients, as evidenced by a higher prevalence of both obstructive and non-obstructive lesions (Koulaouzidis *et al*, 2013). This coincided with higher coronary artery calcification (CAC) in the South Asians groups. Caucasians patients below the age of 50 yrs had more diffuse disease than South Asians (Koulaouzidis *et al*, 2013). Furthermore, a study by Tilin et al revealed that South Asians had more proximal left anterior descending (LAD) stenoses than Europeans for similar coronary artery calcium (CAC) scores. In the South Asians, there was a predilection for disease in the proximal LAD than Caucasians for all CAC scores (Tillin *et al*, 2008). In addition, there was evidence that South Asians had narrower LAD diameters, which predisposed them to more disease than Caucasians. Tilin et al revealed that South Asians in the lowest CAC tertile had narrower LADs than Caucasians; however, this trend disappeared for the higher tertiles (Tillin *et al*, 2008).

The mortality from CAD was significantly higher in the South Asians compared to the Caucasians (McKeigue, Miller and Marmot, 1989). This has been explored in more contemporary datasets. Jones et al investigated case fatality in South Asian and Caucasian patients after PCI for MI or angina using the large BCIS-NICOR database (Jones *et al*, 2014). South Asians had CAD at an earlier age and more risk factors compared with Caucasians patients, however, the unadjusted all-cause mortality rates after PCI were better in the South Asians compared with the Caucasians. Subgroup analysis revealed that Caucasians fared worse after primary PCI than South Asians. Cox analysis revealed higher mortality in the South Asian group compared with Caucasians, however after correction for the numerous factors such as diabetes, chronic total occlusions (CTO) and chronic kidney disease (CKD), abolished the differential mortality. Counter intuitively, South Asians had higher all-cause mortality after elective PCI than Caucasians (Jones *et al*, 2014). A study by Toor et al reported that South Asians have greater re-stenosis rates than

Caucasians after PCI for the index coronary artery lesion, requiring further PCI and coronary artery bypass grafting (CABG) (Toor *et al*, 2011). However, this observation did not translate to an increased mortality for the South Asians compared to the Caucasians (Toor *et al*, 2011). Such PCI procedures were performed at the beginning of the century (2002-2004), before the mainstream use of drug eluting stents. Hence, the use of first generation stents along with their inherent restenosis rates may have been influenced by the patient's diabetic status to a greater extent than the current stents (Toor *et al*, 2011).

6.2 Hypothesis

Lipoproteomic differences exist between South Asians and Caucasian patients with CAD and such differences may be utilised to establish risk.

6.3 Aims

The aim of this study was to explore the lipoproteomic differences between South Asian and Caucasian patients with CAD to identify novel markers that confer risk. In addition, to identify novel proteins that may give insight into mechanisms that initiates and promotes disease progression. The novel lipoaffinity resin was used to reliably isolate the lipoproteins and their associated protein cargoes and then subjected to the typical proteomic workflow, with ultimate analysis on the high definition ion mobility enabled MS for enhanced peptide separation.

6.4 Materials and Methods

6.4.1 Materials and reagents

All materials and reagents that were used are described in Chapters 2 and 3.

6.4.2 Patient Recruitment

Patients with known CAD were recruited into this prospective cohort study. This study complied with the declaration of Helsinki and was approved by the local ethic committee (REC reference: 13/EM/0049). Patients were recruited after a minimum of 3 months post ACS event and after a minimum of 1 month of statin stabilisation. Written informed consent was obtained from all patients. A questionnaire regarding the patient's medical history and medications was collated and confirmed with the medical notes. Blood pressure was recorded three times in resting patients using calibrated sphygmomanometers. Waist and hip measurements were recorded. Patient's height and weight measurements were recorded on a calibrated stadiometer. Blood was withdrawn from consented patients and stored in pre-chilled 30ml Universal containers containing 80µL 1M EDTA (Sigma Aldrich) in 2M NaOH & 250 µL Trasylol (Nordic Pharma) per 20 mL of blood. Blood was centrifuged at 1500 G for 15minutes at 4 °C, the plasma was aliquoted into eppendorfs and stored in a -80 °C until analysis. Patients with a known history of heart failure, ejection fraction below 45 %, renal disease (eGFR below 50) or inflammatory diseases were excluded from this study. Modified diet in renal disease formula was used to calculate glomerular filtration rate (Stevens *et al*, 2007).

6.4.3 Angiographic analysis

The coronary angiograms for the patients were assessed by a cardiologist who was blinded to their risk factors and diagnosis. Significant CAD was defined as a coronary luminal stenosis greater than 50% in either a one vessel, two vessels or three vessels or the major branches of the coronary arteries.

6.4.4 Sample Preparation

Plasma samples were randomised prior to sample preparation and prepared in batches of 20. Samples were prepared blinded to clinical details. The sample preparation is described in section 3.3.4.7. A pooled QC consisting of 10 µL from each patient sample was created. The pooled QC was used to ensure platform

reliability and consistency. The prepared samples were randomised again prior to LC-MS/MS injection to overcome batch variability. Samples were reconstituted in 0.1 % FA and spiked with an equal volume of 100 fmol of ADH (Waters Ltd.). Reconstituted samples were run in batches of 10. Prior to each batch, 1 µL of HeLa protein digest standard (Thermo Fisher Scientific Ltd) and 1.4 µL of QC were injected on the LC-MS/MS (Waters Ltd.) to ensure optimal performance of the MS. A combination of HeLa standard, yielding over 2000 protein hits and QC protein hits within 10 % were deemed satisfactory for the running of the subsequent batch. Initially 1µl scouting injections for each sample were performed over a 75 minute gradient. Triplicate injections were performed for each sample over a 110 minute gradient in HDMS^E. Each sample injection loaded 500 ng onto the analytical column.

6.4.5 Protein Identification

PLGS threshold inspector (Sourceforge; www.sourceforge.net/projects/plgsthresholdin/) was used to determine the optimal protein hits for the samples. MS^E runs were analysed using the best threshold of 300-10-750. The amount loaded on the column for the 1 µL scouting runs were calculated and the volumes were adjusted to ensure that 500 ng was loaded onto the column. Threshold inspector was employed to optimise the raw data processing so as to achieve the maximum high confidence protein identifications in a short comparable time window (60-65mins) Figure 6.1. The optimised threshold was 100-10-750 which was used for subsequent analysis of the QC injections between the batches. Using this threshold posed a significant challenge for the processing computers, as this stringent criteria consumed up to 7 hours of analysis time per sample. It was therefore decided to use less stringent threshold for analysing the triplicate runs, in order to improve throughput. From Figure 6.1, it was evident that least stringent threshold was 350-50-750, still identified 40 protein hits, only 10 below the optimal threshold. Samples were therefore analysed using 350-50-750 with a FDR of 1%.

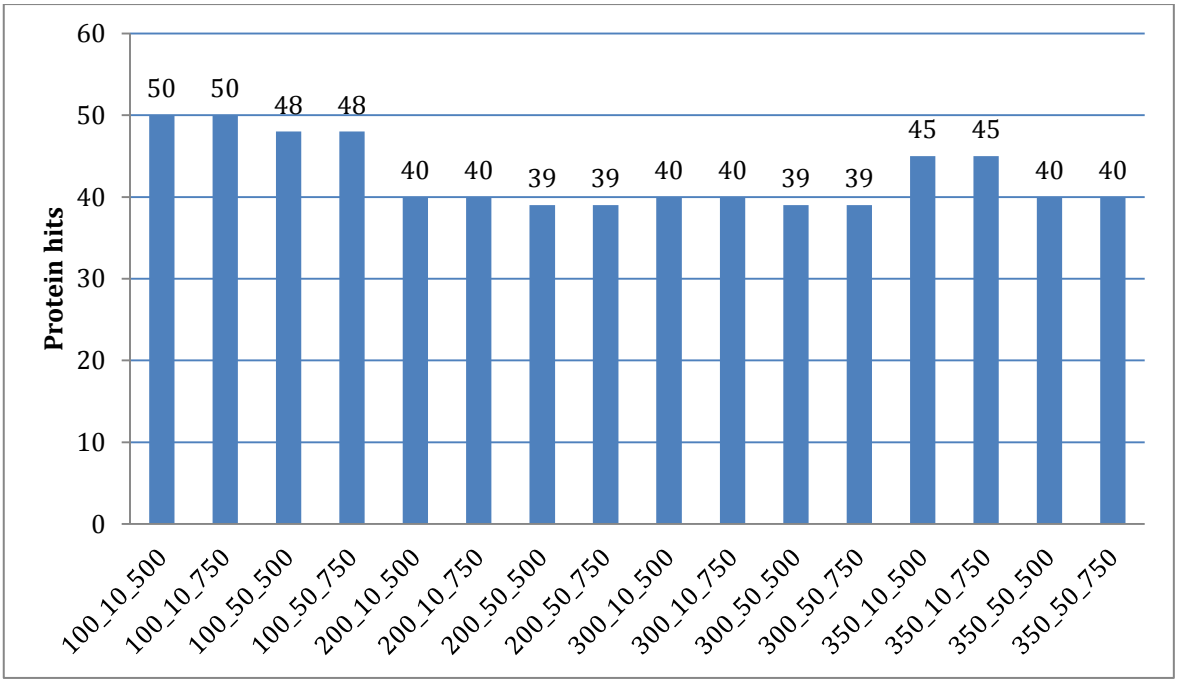


Figure 6.1 Bar chart showing the protein hits obtained using different permutations of low energy, high energy, and intensity thresholds for a single QC sample.

6.4.6 Progenesis

Differential protein expression was analysed using Progenesis QI software (Nonlinear Dynamics). The optimal threshold was set to 100-10-750. The raw data was aligned using the QC1 run to allow for composite comparison between the different groups. Fixed modification of carbamidomethylation and variable modifications consisting of deamidation N, deamidation Q, oxidation M, Phosphoryl STY were selected for protein identification. A stringent FDR of less than 1 % was chosen. For peptide matching 2 or more fragments were required. 5 fragments were required for protein identification and 2 peptides required for protein identification. For protein quantification, Hi-3 was used. Triplicate injections of the same sample were analysed for all patients.

6.4.7 Statistical analysis

Statistical analysis was performed using SPSS version 22 (IBM, US). Continuous variables were analysed using the independent t-test. Categorical variables were analysed using Chi squared with continuity correction. The relationship between

two continuous variables was assessed using the Spearman Rho correlations. Scatter graphs were constructed to display the relationship between two continuous variables. Boxplots were used to show the trend between continuous variables and discrete variables, with the line representing the median value and the whiskers of the boxplot representing the 25th and 75th centiles. Stepwise linear regression was used to identify the independent predictors for the dependent variable. Variables that were entered into this multivariate model were variables that were related to the dependent variable in univariate analysis ($P<0.1$) or other variables known to influence it. For the purposes of this study variables that were entered into multivariate analysis included; age, history of MI, type of MI, history of diabetes, history of hypertension, history of hypercholesterolemia, smoking history, aspirin use, β blocker use, statin use, ACE inhibitor/ARB use, systolic blood pressure, heart rate, waist hip ratio, BMI, total cholesterol, HDL, LDL, triglycerides, urea, creatinine, eGFR MDRD and number of diseased vessels on angiogram. Continuous variables were expressed as mean ± SD, categorical variables expressed as percentages. A P value below 0.05 was deemed statistically significant.

6.5 **Results**

6.5.1 Clinical demographics

The demographics and clinical data for this cohort are presented in

Table 6.1. All participants recruited into this study were male. The South Asians comprised predominantly of those individual of Indian heritage (84 %). South Asians were significantly younger than the Caucasians (58±8.57 vs. 64±8.69; P=0.001 respectively). Diabetes mellitus was more prevalent in the South Asian cohort than the Caucasian cohort (39.2 % vs. 12.2 %; P=0.002) consistent with previous findings (McKeigue, Shah and Marmot, 1991). The rates of hypertension and hypercholesterolemia were comparable between the two groups. The majority of patients had a previous history of myocardial infarction with similar rates of PCI. The prevalence of CABG was comparable between the South Asians and the Caucasians (15.7 vs. 4.1; P=0.110) respectively. Heart rate was significantly lower

in the Caucasians than the South Asians (59.82 bpm ±7.79 vs. 64.73±11.31; P=0.013 respectively). There was no significant difference in mean blood pressures between the two cohorts. BMI was significantly lower in the South Asian patients compared with the Caucasians patients (27.22 kg/m^2 ± 4.04 vs. 30.38 ± 4.55; P<0.001), despite comparable waist hip ratios. The prescription of the secondary medications was comparable between the South Asians and Caucasian patients with CAD. Concerning the lipid parameters, there was no significant difference in total cholesterol, LDL, HDL or triglycerides between the two groups, a reflection of similar statin use between the cohorts. There was no significant difference in the total number of coronary vessels affected with CAD between the South Asians and the Caucasians suggesting comparable burden of CAD. There was a predilection for CAD to occur in the left circumflex arteries in the South Asians compared to the Caucasians (56.9 % vs. 32.7 %; P=0.026). However, there was no difference in burden of disease in the other coronary arteries for the two ethnicities.

Table 6.1 Demographic and clinical variables for the CAD patients classified according to ethnicity.

	South Asians n=51	Caucasians n=49	P value
Age	58±8.57	64±8.69	0.001
Gender (% male)	100	100	
Diabetes (%)	39.2	12.2	0.004
Hypertension	51.0	42.9	0.540
Hypercholesterolemia	41.2	51.0	0.431
Current smoker (%)	5.9	6.1	1.000
Former smoker (%)	37.3	67.3	0.005
Never smoked (%)	60.8	26.5	0.001
Previous MI (%)	90.2	91.8	1.000
Previous CABG (%)	15.7	4.1	0.110
Previous PCI (%)	92.2	93.9	1.000
PVD	0	2	0.984
Previous CVA	0	2	0.984
Type of infarction			
STEMI (%)	64.4	74.4	0.434
Physical Characteristics			
Heart rate (bpm)	64.73±11.31	59.82±7.79	0.013
SBP (mmHg)	135 ± 16.68	140 ± 17.35	0.124
DBP (mmHg)	78 ± 11.55	80 ± 10.51	0.294
BMI (kg/m^2)	27.22 ± 4.04	30.38 ± 4.55	<0.001
Waist hip ratio	1.02 ± 0.05	1.00 ± 0.06	0.104
Biochemical data			

Urea (mmol/L)	5.78 ±1.73	6.25± 1.13	0.108
Creatinine µmol/L	80.53±17.19	84.06±14.26	0.267
eGFR_MDRD	95.81 ± 21.75	88.29± 18.83	0.068
Total cholesterol (mmol/L)	3.66 ±0.92	3.68 ± 0.80	0.905
LDL (mmol/L)	1.79 ± 0.68	1.73 ± 0.72	0.675
HDL (mmol/L)	1.14 ± 0.35	1.12 ± 0.23	0.718
Triglycerides (mmol/L)	1.73 ± 1.20	1.89 ± 1.08	0.489
Medications			
Aspirin (%)	96.1	89.8	0.402
B blocker (%)	86.3	89.8	0.815
ACEi/ARB (%)	92.2	91.8	1.000
Statin therapy (%)	92.2	98.0	0.383
Number of diseased vessels			
Single-vessel (%)	54.9	55.1	1.000
Multi-vessel (%)	45.1	44.9	1.000
Coronary artery affected			
RCA (%)	49	63.3	0.218
LAD (%)	47.1	61.2	0.222
Cx	56.9	32.7	0.026

6.5.2 Platform Reproducibility

1 µL injection of HeLa cell was run prior to each batch, loading 500 ng onto the column. A cut-off of 2000 or more proteins hits was required in order to proceed to analysing the pooled QC. The protein hits for the HeLa cell are present below in Figure 6.2. The CV in protein hits between batches was only 4.82 %, indicating a consistent platform performance. The total number of peptides for each HeLa cell injection showed good consistency, with an impressive CV of 5.09 % Figure 6.2.

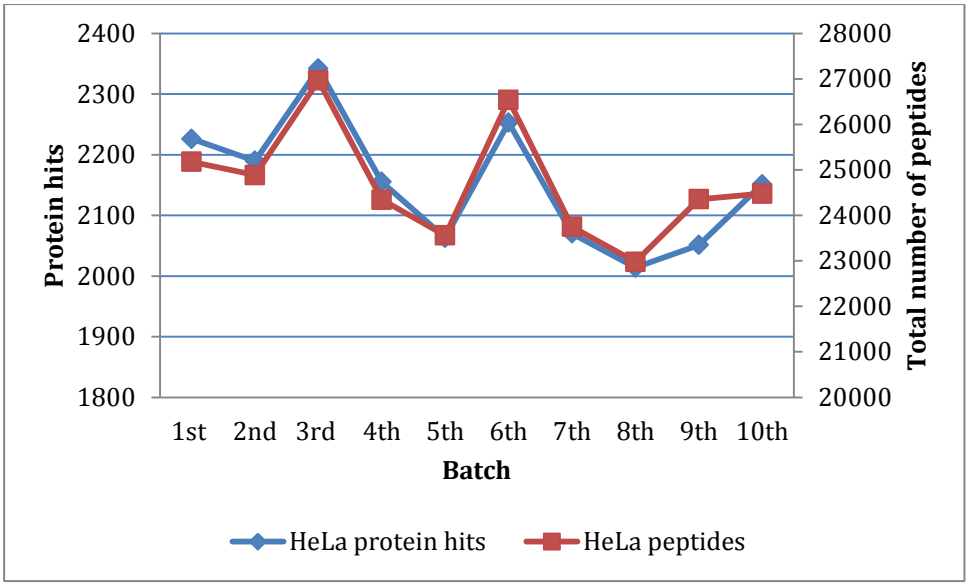


Figure 6.2 Protein hits and total number of peptides identified for the HeLa cell lysate injection performed prior to each respective batch. Protein hits and the total number of peptides for the injection were consistent during the samples injections indicating reliable data acquisition.

In addition to the HeLa injection, a 1.4 µL injection of the pooled QC was analysed on the LC-MS/MS prior to batch analysis, for further robustness and confidence in platform reliability. The injection of 1.4 µL loaded 500 ng onto the analytical column, with the insight that the resulting chromatogram would potentially be used as a reference run for sample alignment in Progenesis QI. The protein hits for each QC are presented in Figure 6.3. The mean protein hits for the QCs across all batches was 130.8 ± 6.65, yielding a CV of 5.08 %. The individual peptides for each QC injection were analysed which also showed good reliability, Figure 6.3. The mean peptides identified for the QCs injections across the batches was 2920.70 ± 232.80 with a CV of 7.97 %, Figure 6.3. The total number of peptides for a variety of low, medium and high abundant proteins across all ten pooled QC injections was analysed Figure 6.4 .The narrow error bars representing the standard deviation in total peptides, indicated good confidence for the high and low abundant proteins.

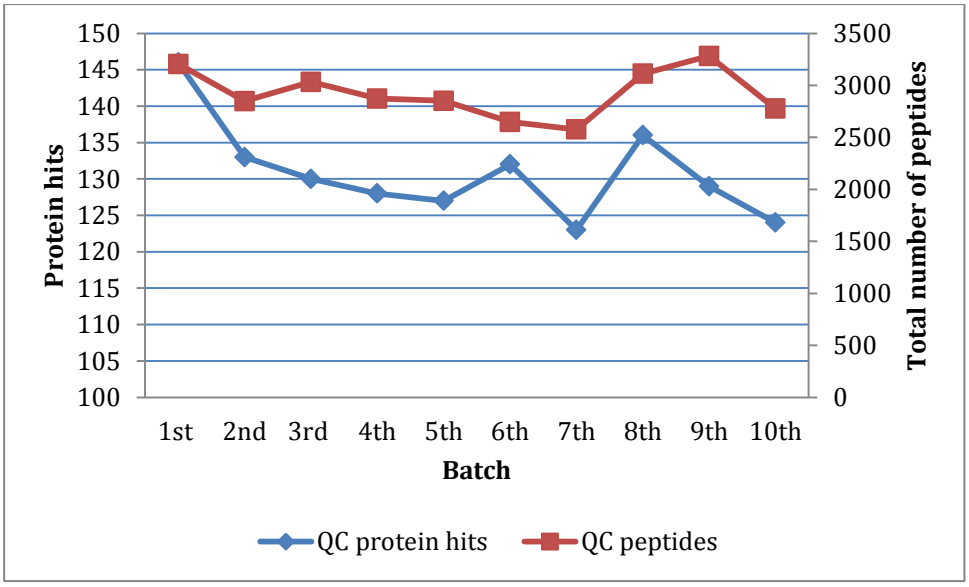


Figure 6.3 Protein hits and total number of peptides identified for the pooled QC injection performed prior to each sample batch. The coefficient of variation in protein hits and for total number of peptides were 5.08% and 7.97% respectively, indicating reliable and consistent data acquisition.

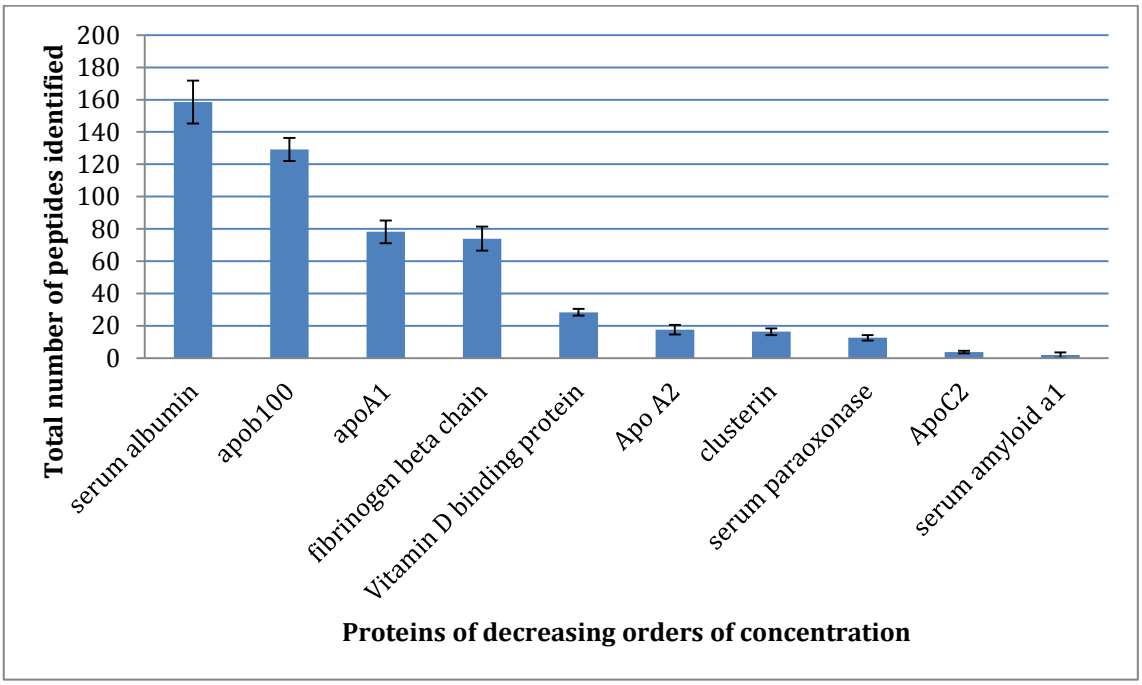


Figure 6.4 Bar chart showing the total number of peptides identified for a variety of high, medium and low abundant proteins across all ten pooled QC injections. The narrow error bars indicate good confidence for the high and low abundant proteins. The bars represent the mean and the error bars represent the SD.

Analysis of the extracted ion chromatograms for m/z 682.72 revealed minimal drift in retention times over the course of the study, reinforcing the validity and reliability of this mass spectrometry platform Figure 6.5.

Figure 6.5 Extracted ion chromatogram for ions with a m/z 682.72 in the ten pooled QC samples. The mean retention times for the ions was 92.89mins (SD±0.64) indicating minimal drift over the ten batches

6.5.3 Protein identification

The mean protein hits for the entire cohort of patients were 123.63 ± 7.77. The mean CV for the proteins hits for all the patient samples was 4.80 % ± 2.57. Analysing the samples further revealed that there was no significant difference in the mean protein hits between the South Asian and Caucasian patients with CAD (122.91 ± 7.87 vs. 124.38 ± 7.67; P=0.348) Figure 6.6. In addition, there was no significant difference in the CV for the protein hits between the South Asian and Caucasian patients (5.08 % ± 2.65 vs. 4.50 ± 2.47; P=0.259) Figure 6.7.

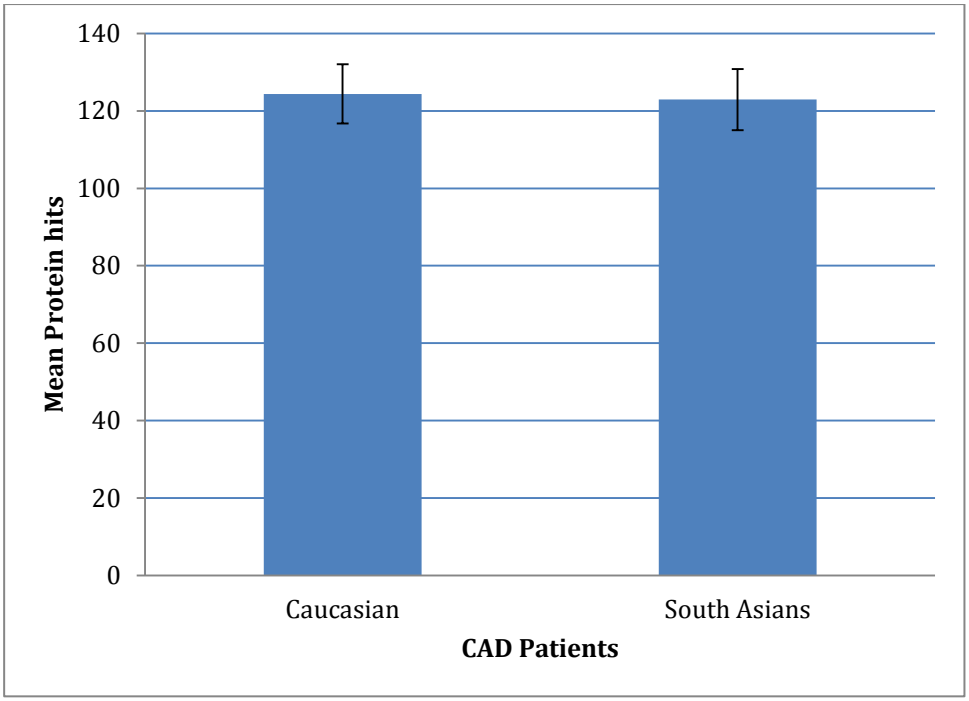


Figure 6.6 Bar chart showing the protein hits for the South Asians patients (n=51) and Caucasian patients (n=49) with CAD. Bars represent the mean protein hits and the error bars represent the SD (P=0.348).

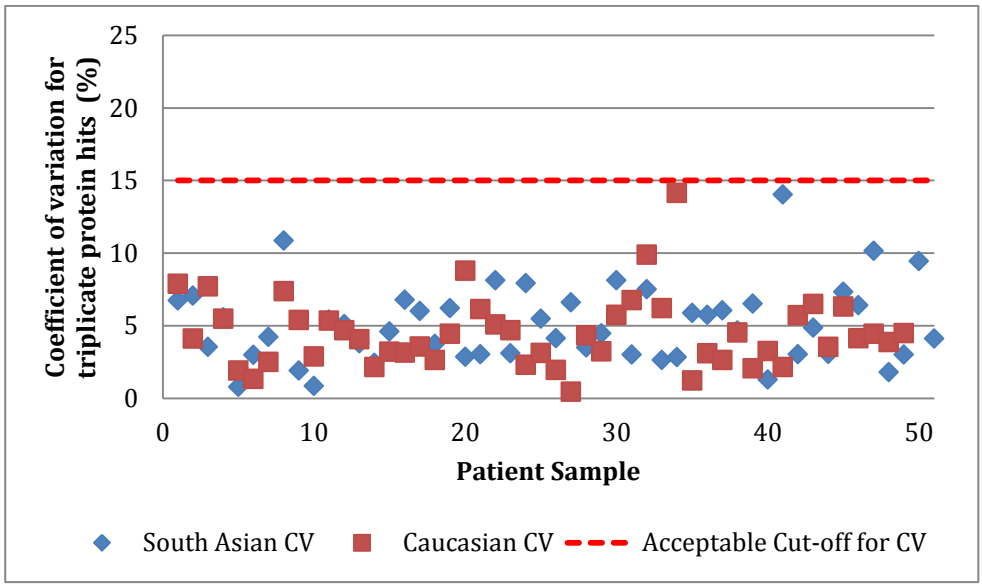


Figure 6.7 Scatter graph showing the coefficient of variation in the protein hits for each triplicate patient sample injection classified according to ethnicity. The majority of the samples had a coefficient of variation below 10% indicating consistency between the triplicate injections.

6.5.4 Protein Expression

Two hundred and seventy two proteins were identified using the progenesis QI software. Of those, 28 proteins demonstrated significant differential expression between the South Asians and Caucasian patients with CAD ($P<0.05$) Table 6.2 & Figure 6.8.

Table 6.2 Proteins demonstrating significant differential expression between the South Asian and Caucasian patients with CAD.

Protein	Mean South Asian protein intensities	Mean Caucasian protein intensities	Fold change (Caucasian vs. SA)	T Test
Ig kappa chain C region	395656.98 ± 155198.70	313732.35 ± 109564.60	1.26	0.003
Cartilage acidic protein 1	481.54 ± 154.68	596.84 ± 223.58	0.81	0.004
Ig gamma-4 chain C region	96366.62 ± 40772.78	78202.64 ± 18846.68	1.23	0.005
Carboxypeptidase B2	40981.67 ± 15694.70	33752.79 ± 9450.35	1.21	0.006
Ig kappa chain V-I region Roy	7039.92 ± 2432.97	5891.40 ± 1750.56	1.19	0.008
Ig kappa chain V-III region GOL	4794.11 ± 2132.00	3821.38 ± 1441.04	1.25	0.009
Plasma serine protease inhibitor (SERPINA5)	13896.87 ± 3661.98	12170.29 ± 2782.63	1.14	0.009
Ficolin-2	12259.83 ± 6249.72	9672.25 ± 2919.25	1.27	0.009
Ig heavy chain V-II region OU	1065.71 ± 486.73	790.98 ± 571.25	1.35	0.011

Brain acid soluble protein 1	194.35 ± 251.34	96.35 ± 99.44	2.02	0.012
Extracellular matrix protein 1	39730.31 ± 15546.09	33287.61 ± 9680.39	1.19	0.014
Ig gamma-1 chain C region	337597.81 ± 144267.60	281948.63 ± 72550.43	1.20	0.017
Nck-associated protein 5-like	14569.03 ± 5377.67	12265.49 ± 4096.30	1.19	0.018
Haptoglobin	183967.06 ± 65134.44	155466.20 ± 56805.13	1.18	0.022
Tetranectin	12116.79 ± 4546.45	14140.54 ± 4425.04	0.86	0.026
Thrombospondin -1	928.18 ± 708.85	672.19 ± 380.52	1.38	0.027
Platelet factor 4	2875.68 ± 2737.39	1867.48 ± 1601.48	1.54	0.027
Tubby-related protein 2	6179.12 ± 2000.62	5385.28 ± 1528.93	1.15	0.028
N-acetyl-muramoyl-L-alanine amidase	37181.17 ± 13529.78	45128.63 ± 21594.27	0.82	0.031
Ig heavy chain V-III region BUT	868.97 ± 852.42	1313.12 ± 1140.18	0.66	0.030
Ig kappa chain V-II region Cum	28738.68 ± 11860.10	23320.55 ± 12786.95	1.23	0.031
Alpha-1-acid glycoprotein 2	8613.37 ± 2233.32	7724.03 ± 1864.44	1.12	0.033
Profilin-1	694.29 ± 400.08	557.67 ± 204.81	1.24	0.034

GXP 3	5746.25 ± 2783.28	4687.06 ± 2162.35	1.23	0.036
Centriole, cilia and spindle-associated protein	4183.31 ± 1863.16	3552.76 ± 983.32	1.18	0.037
Complement factor H-related protein 1	196973.58 ± 66631.22	170341.89 ± 61859.65	1.16	0.041
Hepatocyte growth factor-like protein	3825.26 ± 953.81	3463.88 ± 792.55	1.10	0.042
Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H3	4304.07 ± 1494.65	3737.17 ± 1258.06	1.15	0.043

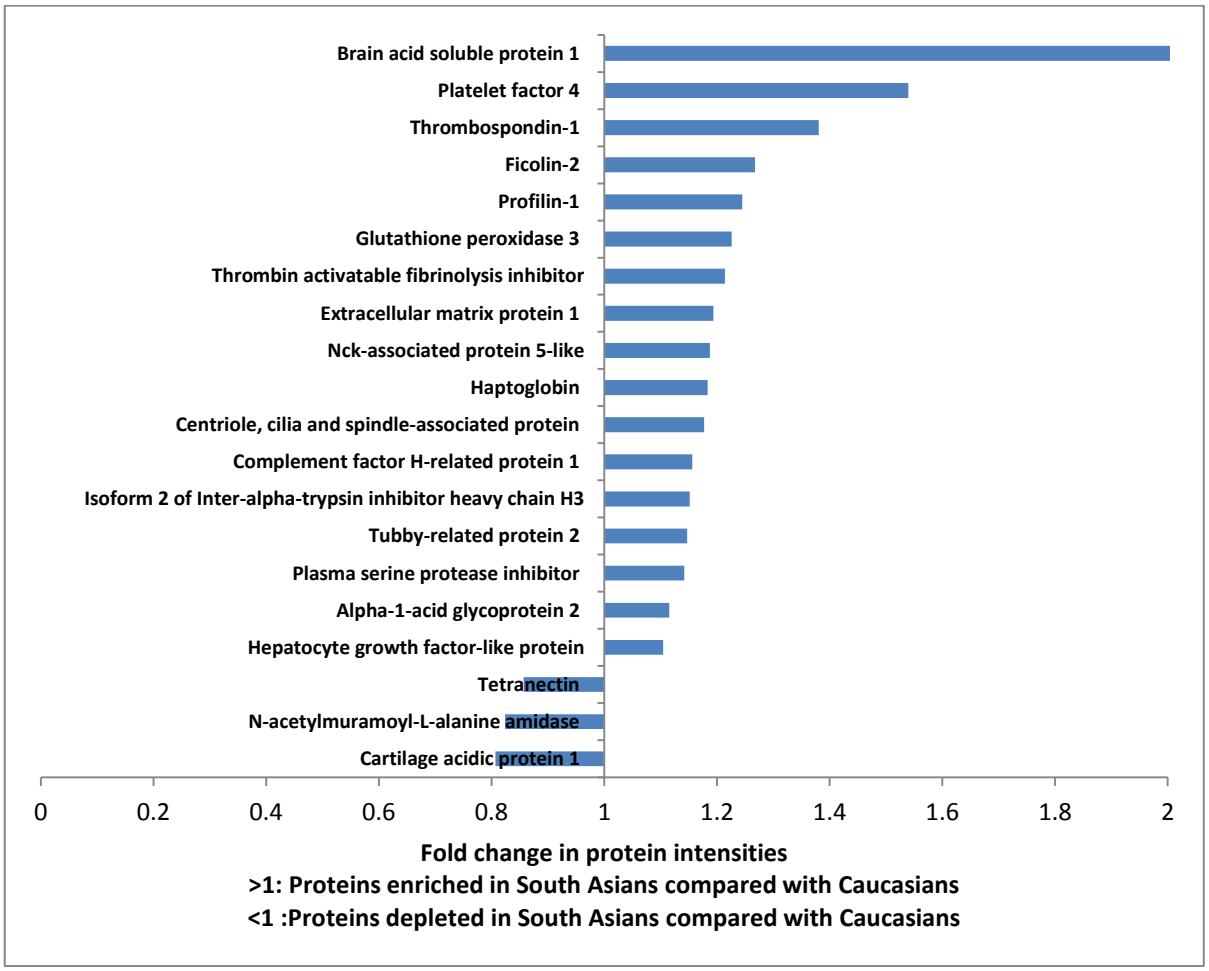


Figure 6.8 Bar chart showing the fold change in protein intensities between the two ethnicities. Fold change >1 indicated protein enrichment in the South Asian patients. Fold change <1 indicates protein depletion in the South Asian cohort. Data derived for triplicate analysis in Progenesis QI for Proteomics. Data subject to t-test analysis.

6.5.5 Correlations

Spearmans correlations were performed to assess the relationship between the laboratory determined lipid levels and the protein intensities. There was a significant correlation between HDL levels and apoA1 (rs=0.255, p=0.011) and apoA2 (rs=0.248, p=0.013) intensities, Figure 6.9. This association is expected as both apoA1 and apoA2 are the main constituent apolipoproteins in the HDL particle. This observation also reinforces that the observed protein intensities are likely to be a genuine reflection of plasma rather than a by-product of sample processing. No relationship was observed between LDL levels and apoB100 intensities (rs=-0.106, p=0.304) Figure 6.10. The lack of association between the two variables may be due, in part, to the fact that LDL is calculated using the Freidewald formula rather than directly measured. Furthermore, the unbiased assessment of the lipoproteomic cargo, may have resulted in a larger apoB100 signal, reflective of the other apoB100 containing lipoproteins and therefore minimised any association with LDL levels. There was no significant difference in apoA1 intensities (561355.68± 327519.24 vs., 548843.83 ± 296375.07; P=0.842) and apoB100 intensities (326583.09 ± 104040.15 vs. 308182.13 ± 90618.31; P=0.349) between the respective South Asian and Caucasian patients in this study. This is consistent with the lack of any difference in the laboratory determined HDL and LDL levels between the two ethnicities.

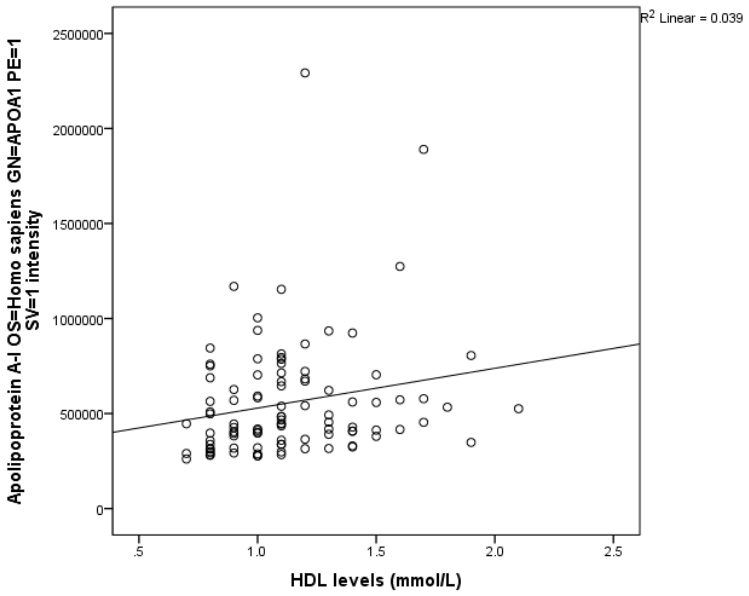


Figure 6.9 Scatter graph showing the relationship between HDL levels and apoA1 intensities rs=0.255, p=0.011).

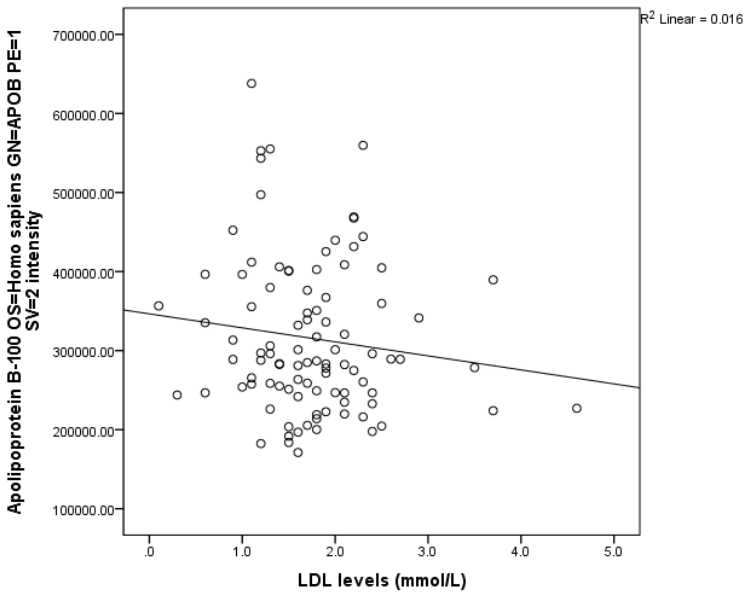
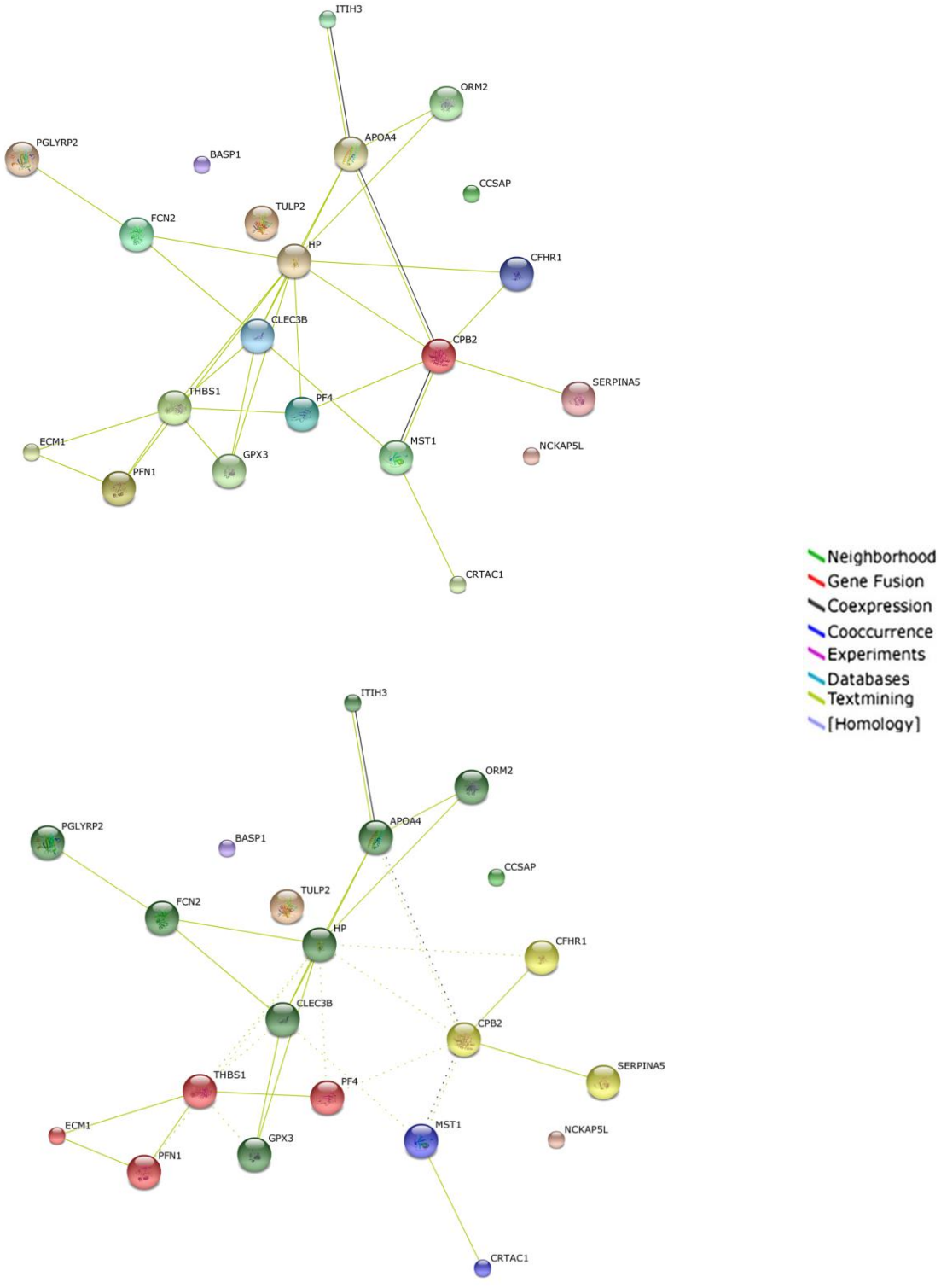


Figure 6.10 Scatter graph showing the relationship between LDL levels and apoB100 intensity (rs=-0.106, p=0.304).

6.5.6 Pathway Analysis

The differentially expressed proteins between the South Asian patients and the Caucasian patients were entered into STRING software to establish protein interactions, Figure 6.11 (A)(Szklarczyk *et al*, 2015). Clustering analysis revealed 3 main functional protein clusters, Figure 6.11 (B). The main functional clusters centred on haptoglobin, carboxypeptidase B2 and thrombospondin 1.

A)



B)

Figure 6.11 Protein-protein interaction networks visualised by STRING. A) Protein-protein interaction networks of the differential expressed proteins in the South Asian patients compared to the Caucasian patients. B) Clustering of the protein interaction networks. The coloured lines represent the different levels of evidence for the interactions.

6.6 Discussion

6.6.1 Cartilage acidic protein 1

The concept of atherosclerosis has evolved to encompass a dynamic multifaceted process. The role of vascular calcification in its aetiology is of growing importance. The calcification of the arteries maybe initially a protective response to inflammation, but eventually culminates in pathological calcification of the vascular tree, promoting arterial stiffness. Chondrocytes and osteoblasts have an integral role in vascular calcification and are influenced by an over-expression or an under-expression various bone mineral signals such as alkaline phosphatase, collagen 2 and osteocalcin or aggrecan and osteonectin, respectively (Tyson *et al*, 2003). In this study, cartilage acid protein 1 was up-regulated in the Caucasians CAD patients compared with the South Asian CAD patients (mean protein intensity 33752.79± 223.59 vs. 481.54± 154.68; p=0.004) respectively. Chondrocytes and osteoblasts arise from the differentiation of the pluripotent stem cells (Abedin, Tintut and Demer, 2004). Steck et al. demonstrated that cartilage acid protein 1 was exclusively expressed in cultured chondrocytes enabling distinction between the different cell lines and possessed an epidermal growth factor motif responsible for the formation of extracellular matrix (Steck *et al*, 2001). The presence of cartilage acid protein 1 may indicate the presence of chondrocytes in the vascular smooth muscle cells, which may then become engulfed by osteoblasts, resulting in the secretion of bone matrix (Yang *et al*, 2014). Hence cartilage acid protein 1 may be a precursor to vascular calcification. Therefore, the elevated levels in the Caucasian patients may reflect this process to a greater extent than that in the South Asians patients. The presence of vascular calcification may not simply be related to ethnicity, but rather to known variables such as diabetes, ageing, dyslipidaemia, renal failure and statin use (Callister *et al*, 1998). In view of these potential confounders, linear regression was performed to identify the independent determinants of plasma cartilage acidic protein 1. The only independent predictors for cartilage acid protein 1 levels were age (β 0.416 [CI 4.305 to 11.564]; P<0.001) and waist hip ratio (β-0.263 [CI -1409.652 to -225.490]; P=0.007). Therefore, the higher levels observed in the Caucasians was likely to be confounded by their significantly higher age compared with the South

Asians patients. Cartilage acidic protein 1 may potentially represent a surrogate marker for arterial calcification.

6.6.2 Carboxypeptidase B2

In this study carboxypeptidase B2 was up-regulated in the South Asian patients compared to the Caucasian patients with CAD (mean ± SD 40981 ± 15694.70 vs. 33752.79 ± 9450.35; P=0.006). This protein, also known as thrombin activatable fibrinoylsis inhibitor (TAFI), is secreted by the liver as a zymogen. Upon activation by thrombin, carboyxpeptidase B2 cleaves the C-terminal of the fibrin particle, inhibiting the binding of plasminogen to the fibrin surface and preventing its interaction with tissue plasminogen activator, thus impairing fibrinolysis (Bouma *et al*, 2001). Theoretically, elevated levels of TAFI would support a pro-thrombotic tendency; however the results from clinical studies have been conflicting. In stable CAD patients, precursor carboxypeptidase B2 protein levels were higher compared to controls (Silveira *et al*, 2000). Furthermore, intracoronary levels of carboxypeptidase B2 were higher in stable CAD patients compared to control subjects with levels correlated with total cholesterol and fibrinogen (Schroeder *et al*, 2002). Surprisingly, geographical variations in carboxypeptidase B2 levels were observed between French and Irish patients. French patients with high baseline TAFI levels had increased risk of developing angina at 5 years compared to controls, with no association identified for the Irish patients (Morange *et al*, 2003). It was later revealed however, that the higher carboxypeptidase B2 levels were associated with improved survival, with reduced risk of death or MI, but the association disappeared after the correction of conventional risk factors (Juhan-Vague, Morange and PRIME Study Group, 2003).

Plasminogen activating inhibitor 1 (PAI-1), which inhibits fibrinolysis has previously been shown to be higher in South Asians compared to Caucasians, which contributes to their pro-thrombotic state (Anand *et al*, 2000a). It was suggested that in diabetic patients with metabolic syndrome and hypercholesterolemia, elevated levels of both TAFI and PAI-1 cooperate together in promoting impaired fibrinolysis (Aso *et al*, 2005). In multivariate step

regression analysis, the independent predictors for carboxypeptidase B2 levels were LDL levels (β 0.366 [CI 3227.900 to 10761.334]; P<0.001) and South Asian ethnicity (β 0.229 [CI 847.747 to 11354.409]; P=0.023). South Asian ethnicity in the presence of CAD is independently predictive of higher levels of carboxypeptidase B2, which may contribute to a pro-thrombotic state independent of their diabetic status. The independent association between LDL levels and carboxypeptidase B2 is consistent with findings by Aso et al. in diabetic patients, possibly as a potential carrier particle in plasma (Aso *et al*, 2005).

6.6.3 Ficolin-2

The complement pathway is complex and consists of the classical, alternative and lectin pathways, which all converge to activate C3, mediating downstream inflammation. Ficolin-2 is synthesised by the liver and interacts with mannan binding lectin serine protease 1,2,3 (MASP) which in turn activates the lectin pathway (Matsushita, Endo and Fujita, 2000). In this study ficolin-2 levels were significantly higher in the South Asian patients compared to the Caucasian patients (12259.83 ± 9672.25 vs. 9672.25± 2919.25; P=0.009). There was no significant difference in the downstream activators MASP-1 or MASP-2 between the two ethnicities. Interestingly, MASP-2 has been shown to display differential expression depending on the clinical presentation, with acute MI patients having lower levels compared with controls and stable CAD patients (Zhang *et al*, 2013a). In this study, no significant difference in C3 levels was observed between the two ethnicities. However, levels of C3 and the terminal complement complex have been previously shown to be elevated in South Asians compared to Caucasians (Siezenga *et al*, 2009). South Asians with diabetes had higher levels of these complement factors compared to those without diabetes, emphasising the role of diabetes in mediating subclinical inflammation (Siezenga *et al*, 2009).

In a step wise linear regression model, the independent determinants for plasma ficolin-2 levels were total number of diseased vessels (β 0.227 [CI 175.572 to 3568.549]; P=0.031) and South Asian ethnicity (β 0.251 [CI 460.298 to 4882.491]; P=0.019). Hence, the elevated levels of ficolin-2 in the South Asians may contribute

towards or reflect a greater degree of subclinical inflammation compared to the Caucasians.

6.6.4 Brain acid soluble protein 1

In this study, a novel protein called brain acid soluble protein 1 (BASP-1) was up-regulated in the South Asian patients compared to the Caucasian patients with CAD (194.35± 251.34 vs. 99.35 ± 99.44; P=0.012). BASP-1 is expressed in endothelial cells, kidneys, axontal termini, testis, and lymphoid tissue (Mosevitsky *et al*, 1997). It was recently shown that BASP-1 was a regulator of apoptosis in renal tubules in diabetic nephropathy. Renal tubular cells exposed to stimuli known to induce apoptosis such as high glucose had higher expression of BASP-1. Furthermore, the under-expression of BASP-1 conferred protection to apoptosis inducing stimuli, with consequent reduction in apoptosis (Sanchez-Nino *et al*, 2010). Although the underlying mechanism remains to be elucidated, the modulation of BASP-1 offers an attractive therapeutic target in regulating apoptosis. It is conceivable that the role of BASP-1 as a transcription factor may not be primarily confined to renal tubules, but to other tissues (Mosevitsky *et al*, 1997; Sanchez-Nino *et al*, 2010). The process of atherosclerosis is dynamic and involves apoptotic pathways. The intimal and vascular smooth muscles cells undergo programmed cell death contributing to plaque instability and possible rupture (Geng and Libby, 1995). Risk factors contribute to endothelial dysfunction with consequential induction of apoptotic pathways an antecedent to plaque formation (Hasdai *et al*, 1999). In this study, BASP-1 may reflect atherosclerotic apoptosis and possible plaque destabilisation, the higher levels in the South Asian cohort may be confounded by the higher prevalence of diabetes, an initiator for its secretion, compared to the Caucasians. However, in a stepwise multivariate model the only predictor for BASP-1 levels was increasing heart rate (β 0.451 [CI 4.716 to 11.874]; p<0.001).

6.6.5 Tetranectin

In this study tetranectin intensity levels were significantly lower in the South Asian group compared to the Caucasian group (mean ± SD 12116.79 ± 4546.45 vs. 14140.54 ± 4425.04; P=0.026). Tetranectin consists of 4 identical non-covalently bonded polypeptide chains. The C terminal was discovered to have high affinity for the kringle 4 part of plasminogen, whilst the N terminal has a heparin binding domain (Clemmensen, Petersen and Kluft, 1986). Tetranectin has been shown to be expressed in various tissues from pituitary, pancreas, adrenal medulla and liver, with the latter suggested to be the principal site for synthesis for release into plasma (Christensen *et al*, 1987). It was recently shown that tetranectin bound to plasminogen and tissue type plasminogen activator in a dose dependent manner (Westergaard *et al*, 2003). The result of this interaction was to enhance fibrinolysis, thereby acting as a cofactor (Westergaard *et al*, 2003). In thrombolysis era, tetranectin levels were shown to be lower in acute MI patients compared with control subjects, with levels increasing after clot dissolution. Tetranectin levels were comparable between those acute MI patients who were treated medically and those in whom clot buster drugs failed to re-canalise the coronary artery, inferring an integral role for tetranectin in fibrinolytic pathway (Kamper *et al*, 1998).

However, in this study the comparable rates of PCI and the number of disease vessels between the two ethnicities are likely to suggest equivalence in coronary patency/disease burden. The lower levels of tetranectin in the stable South Asians CAD patients may simply reflect an inherent biological difference in fibrinolysis, contributing to a pro-thrombotic state compared with the Caucasians CAD patients. In a multivariate stepwise linear regression model, South Asian ethnicity (β -0.223, P=0.035) and smoking history (β 0.237, P=0.026) were independently predictive of tetranectin levels.

6.6.6 Thrombospondin-1

In this study thrombospondin-1 (TSP-1) levels were significantly higher in the South Asian CAD cohort compared with the Caucasian CAD cohort (mean ± SD 928.18± 708.85 vs. 672.19 ± 380.53; P=0.027 respectively). TSP-1 is a protein,

primarily concerned with cell-matrix interactions. In a rodent model of diabetes, TSP-1 levels were increased in the intima, media and adventitia of aorta compared to lean controls (Stenina *et al*, 2003). TSP-1 exerts anti-angiogenic effects, demonstrated by a reduction in the vasa vasorum of the aorta in diabetic rats compared to controls, possibly contributing to ischemia and acting as a stimulus for vascular smooth muscle proliferation and thus instigate atherosclerosis (Stenina *et al*, 2003). Furthermore, balloon induced vascular injury was associated with an over-expression of TSP-1 levels compared to controls (Stenina *et al*, 2003). In a murine model of infarction, TSP-1 levels were increased in the border zone between normal myocardium and infarcted myocardium (Frangogiannis *et al*, 2005). TSP-1 may however exert a protective role against pathological remodelling in MI, as TSP-1 knock out rodent models had an enhanced inflammatory response with increased inflammatory cell infiltration and cytokine response culminating in greater fibrotic remodelling (Frangogiannis *et al*, 2005). TSP-1 levels were higher in patients with CAD compared to those without and were independently predictive of CAD in multivariate analysis (Choi *et al*, 2012).

In this study, stepwise multivariate linear regression only identified South Asian ethnicity (β 0.248, P=0.022) as an independent predictor of TSP-1 levels. The higher levels in the South Asians may reflect a greater degree of extracellular remodelling, despite comparable burden of CAD with the Caucasians.

6.6.7 Glutathione Peroxidase 3

The hallmark of atherosclerosis is the peroxidation of lipids. Cells are uniquely equipped with various antioxidants such as GXP, catalase and superoxide dismutase, which protect against oxidative damage (Forsberg, de Faire and Morgenstern, 2001). In this study, South Asians had higher levels of GXP3 compared with Caucasians (mean ± SD 5746.25 ± 2783.28 vs. 4687.06 ± 2162.35; P=0.037 respectively). GXP represents a family of 8 isomers which are primarily concerned with the detoxification of hydrogen peroxide and lipid peroxides (Flohe, 1988). Knockout murine models for ApoE and GXP1, an antioxidant universally expressed in the cytoplasm of cells, showed increased atherosclerotic lesions with

increased smooth muscle cell content, compared with apoE null mice (Torzewski *et al*, 2007). Levels of nitric oxide bioavailability were significantly reduced in the GXP1/Apo knockout mice compared to controls, reinforcing the link between antioxidants and endothelial function (Torzewski *et al*, 2007). GXP4 which is also widely expressed, with a predilection for detoxification of lipid peroxides, was shown to protect against the initiation and progression of atherosclerosis (Guo *et al*, 2008). Furthermore, an over expression of GXP4 in apoE null mice, resulted in reduced atherosclerotic plaques, reduced inflammatory cell adhesion and reduced content of oxidised lipids compared to control apoE null mice (Guo *et al*, 2008). In humans, GXP1 was shown to be protective in CAD, with those in the highest quartile for GXP1 enduring less adverse outcomes compared to those in the lowest quartile (Blankenberg *et al*, 2003).

In this study, GXP3 which is exclusively present in the plasma was found to be differentially expressed between the two ethnicities. In multivariate step wise linear regression, lower BMI (β -0.295, P=0.006) was independently predictive of increasing GXP3 levels in our CAD population.

6.6.8 Alpha 1 acid glycoprotein

In this study alpha 1 acid glycoprotein (AAG) levels were higher in South Asians compared to Caucasians. This protein is an acute phase protein that is synthesised by the liver. It is integrally involved in the binding of various basic drugs in the plasma and thereby influencing their unbound free drug concentration. By contrast, albumin is the main transporter for acidic and neutral drugs in the plasma, with binding via two main sites. The ethnic differences in drug binding and pharmacokinetics were explored in Caucasian and Chinese subjects for a variety drugs (Zhou, Adedoyin and Wilkinson, 1990). The plasma concentration for the free unbound acidic drugs, aspirin and warfarin were similar between the two ethnicities owing to comparable albumin concentrations. In contrast, the free unbound concentrations of the basic drugs propranolol, dispyramide and diphenhydramine were significantly lower in the Caucasians compared with the Chinese subjects. This observation was related to higher level of AAG in the

Caucasians compared to the Chinese subjects (Zhou, Adedoyin and Wilkinson, 1990). The sensitivity of Chinese patients to propranolol is due to higher plasma free drug levels owing to lower AAG levels (Zhou, Adedoyin and Wilkinson, 1990). The higher levels of AAG in the South Asians patients may indicate increased basic drug binding and therefore lower free unbound drug concentration in plasma compared with Caucasians, possibly influencing drug pharmacokinetics. AAG has been previously shown to be related to CAD severity assessed using the Gensini score (Mori *et al*, 1995). Although AAG is an acute phase reactant, the relationship in this study was likely to reflect chronic inflammation as patients within 3months of acute MI were excluded (Mori *et al*, 1995). In a large population based study, using nuclear magnetic resonance spectroscopy, AAG was identified as the strongest independent predictor for all-cause mortality (HR 1.67) at 5 years (Fischer *et al*, 2014). Other independent variables for all-cause mortality included decreasing VLDL particle size, decreasing albumin concentration and increasing citrate levels. The combination of AAG and the above variables were used to create a biomarker score, such that patients in the top quintile had a 19-fold increased risk of all-cause mortality than those in the lowest quintile. It was suggested that the higher AAG levels observed in those that died was likely to represent higher levels of inflammation multiplexed with other systemic disturbances such as lower albumin (renal and liver), VLDL (lipid metabolism) etc (Fischer *et al*, 2014).

In multivariate stepwise linear regression, the only independent predictor for AAG levels was South Asian ethnicity (β 0.236, P=0.030). Higher levels in the South Asians are likely to reflect higher levels of subclinical or chronic inflammation rather than acute inflammation, as there was a recruitment lag of 3months between the ACS episode and entry into this study.

6.6.9 Tubby related protein 2

In this study, a novel protein called tubby related protein 2 (TULP-2) was up regulated in South Asian patients compared to Caucasian patients with CAD (mean ± SD, 6179.12 ± 2000.62 vs. 5385.28 ± 1528.93, P=0.029). This novel protein belongs to the tubby family which have been shown to be intimately involved in

obesity syndrome in rodents. The C-terminal is highly conserved across the animal species and is likely to exert its biological functions rather than the N-terminal which is variable (North *et al*, 1997). The coincidental identification of a link with obesity arose during inbreeding of a colony of mice, which resulted from a splice mutation in the tub gene (Coleman and Eicher, 1990). Homozygous mice were characterised by maturity onset obesity with hyperinsulinemia, which failed to progress to overt diabetes (Coleman and Eicher, 1990). In multivariate stepwise linear regression, the only independent determinant of TULP-2 was South Asian ethnicity (β 0.217, P=0.046). Although, this finding may not directly explain their burden of CAD, it may have a role in predisposing this cohort to an increased risk of obesity, a contributor to their high prevalence of metabolic syndrome.

6.7 **Conclusion**

The burden of CAD in the South Asian community is considerable and serious, owing in part to a huge incidence of metabolic syndrome and diabetes. Although previous studies have shown that this community has higher mortality from CAD, more contemporary databases show that after adjustment for risk factors, their short and medium term mortality was comparable with Caucasians (Jones *et al*, 2014). Despite this comparable mortality, the prevalence of cardiovascular risk factors is greater in the South Asians compared to Caucasians (Jones *et al*, 2014). The interaction of such factors contributes to the initiation and progression of the disease at an earlier age compared with Caucasians. The lipoproteins have been shown to be causally linked with CAD. Although, the evidence supporting increased HDL-C levels in the protection against CAD has been controversial, its functional role is becoming of increasing importance and of pharmaceutical interest (AIM-HIGH Investigators *et al*, 2011; Barter *et al*, 2007a). The lipoproteins are dynamic and contain a unique protein cargo that mediates functions independent of lipid transport which may be protective (Vaisar *et al*, 2007). In the context of CAD, the cargo remodels and becomes dysfunctional (Alwaili *et al*, 2012; Vaisar *et al*, 2010). In this study, we sought to explore the lipoproteins collectively rather than as single entities, as they are likely to operate in unison to mediate

their specific function (Gordon *et al*, 2013). The use of a lipoaffinity binding resin was shown previously to be a reliable and valid method for lipoprotein pull down. The samples were analysed using a high-performance LC-MS/MS equipped with ion mobility for enhanced ion separation, facilitating the discovery of novel proteins. The performance of this platform was constantly scrutinised to ensure reliable and accurate data acquisition. The results of the QCs were impressive lending strength to our findings. Furthermore, the strength of this study derives from the large number of biological samples (n=100) and the three technical replicates per biological sample. This enabled the identification of smaller differences in protein between the two ethnicities with higher precision, principally by overcoming any potential inherent technical variation that is likely to be experienced. In addition, to avoid sample bias and MS analysis bias, samples were blinded and randomised twice and alternated between the two ethnicities. Reassuringly, the protein hits between the two ethnicities were similar. Progenesis QI identified 272 proteins common to both groups with similarities in apoA1 and apoB100 intensities consistent with the lab derived HDL and LDL levels. Twenty eight proteins demonstrated significant differential expression. The South Asian patients had a pro-thrombotic tendency, determined by elevated levels of carboxypeptidase B2 and reduced levels of tetranectin compared with Caucasians. South Asians patients had higher levels of subclinical inflammation as evidenced by higher levels of ficolin 1, which interacts with MASP 1,2,3 to activate the complement pathway, compared with Caucasians. This inflammatory response may be augmented by the higher levels of AAG in the South Asians, which is known to be an acute phase reactant and has recently been shown to be a strong predictor of mortality (Fischer *et al*, 2014). Furthermore, the higher levels of AAG in the South Asians may interfere with drug pharmacokinetics, reducing the free unbound plasma concentration of basic drugs (Zhou, Adedoyin and Wilkinson, 1990). TSP-1 levels were higher in the South Asians compared with the Caucasians, which is likely to reflect a greater degree of extracellular remodelling as a consequence of the multiplicative interaction of the risk factors causing CAD. Such findings may be offset against higher levels of GXP-3 in the South Asians, which protects against oxidative damage. Higher levels of GXP-1 have been shown to confer improved survival compared to those with low levels in ACS patients

(Blankenberg *et al*, 2003). Although, GXP-3 intensities were higher in the South Asians it was confounded by BMI, which was the only independent determinant. A novel protein, TULP-2 was over expressed in the South Asians compared with Caucasians, which may help to potentially explain the high prevalence of obesity and metabolic syndrome in this cohort.

South Asian patients with CAD have an increased burden of CAD compared to Caucasian patients, using a lipoproteomic based approach we have sought to explore and shed light on novel proteins and identified interesting disease mechanisms that may provide the basis for future work.

Table 6.3 The significant differentially expressed proteins that were independently influenced by ethnicity.

	Proteins	Levels in Asians vs. Caucasians
1	Carboxypeptidase B2	↑
2	Plasma serine protease inhibitor (SERPINA5)	↑
3	Ficolin-2	↑
4	Nck-associated protein 5-like	↑
5	Haptoglobin	↑
6	Tetranectin	↓
7	TSP-1	↑
8	TULP 2	↑
9	Alpha-1-acid glycoprotein 2	↑
10	Profilin-1	↑
11	Hepatocyte growth factor-like protein	↑

6.8 Verification

6.8.1 Introduction

Twenty proteins were identified that demonstrated significant differential expression between the South Asian patients and the Caucasian patients with CAD. Of those, certain proteins were independently influenced by ethnicity in multivariate analysis. The proteomic discovery process represents a single facet in the biomarker pipeline. The identification of altered protein intensities between the two ethnicities requires verification to confirm or disprove such associations are genuine and not spurious. Carboxypeptidase B2 and tetranectin were selected for verification in the crude plasma samples, with respective higher and lower levels expected in the South Asians patient samples compared to the Caucasian patients samples, with both proteins contributing to a pro-thrombotic state. The confirmation of such relationships has the potential for real world significance, which may prompt the adoption of more aggressive antiplatelet therapies in those of South Asian ethnicity.

Verification was performed using a single site immunoassay design. Essentially, microtitre plates were coated with crude plasma and incubated overnight. Serial dilutions of plasma were used for generation of the standard curve. The plates were subsequently blocked with BSA at room temperature. Detection was performed using biotinylated antibody, which was added to each well and incubated overnight at room temperature. Plates were incubated with streptavidin and bound chemiluminescence was measured on a Berthold luminometer.

6.8.2 Hypothesis

The excess risk in CAD observed in the South Asian community may be related to dysregulated plasma expression of various pro-thrombotic proteins compared to their Caucasian counterparts. It was hypothesised that plasma levels of carboxypeptidase B2 and tetranectin may be differentially expressed between the two ethnicities, which may favour a hypercoagulable state in the South Asian cohort.

6.8.3 Materials and methods

6.8.3.1 Materials and reagents

All materials and reagents that were used are described in Chapter 2. Mouse anti-human tetranectin (detection) and anti-human carboxypeptidase B2 (detection) antibodies were purchased from R & D systems (Minneapolis, US).

6.8.3.2 Protocol

Day 1

- Plasma stock comprised of 1 in 2000-fold dilution.
- 1 µL of the sample plasma stock was incubated into each microtitre well containing 100 µL of PBS.
- Plasma was used for generation of the standard curve, with a stock solution comprising of 6 µL of plasma diluted in 400 µL of PBS.
- 7 µL of stock solution was added to the top standard containing 1400 µL of PBS.
- Serial 2-fold dilution was performed to generate standards for the calibration curve.

Day 2

- Plates were washed in PBS.
- 150 µL of BSA was added to each well and incubated for 2 hours at room temperature.
- Plates were subsequently washed in solution B.
- Analyte detection was achieved by incubating 20 ng of carboxypeptidase B2 and 20 ng tetranectin antibodies into each respective well.
- Samples were incubated overnight at room temperature.

Day 3

- Plates were washed in solution B.
- 100 µL of biotinylated mouse (1 in 5000 dilution) was added to each well and incubated for 1 hour at room temperature.
- Plates were washed with solution B.
- 100 µL of streptavidin solution was added to each well and incubated in the dark for 90 minutes at room temperature.
- Plates were washed in solution B and bound chemiluminescence was measured on a Berthold luminometer (Berthold, Germany).

The sensitivity (limit of detection) for the carboxypeptidase and tetranectin immunoassays were 729 .55 RLU ± 18.88 and 1292.60 RLU ± 149.48 respectively.

6.8.4 Results

40 South Asian and 40 Caucasian patient samples were analysed blinded to clinical and demographic details. The standard curves for the 2 immunoassays are presented below Figure 6.12 and Figure 6.13.

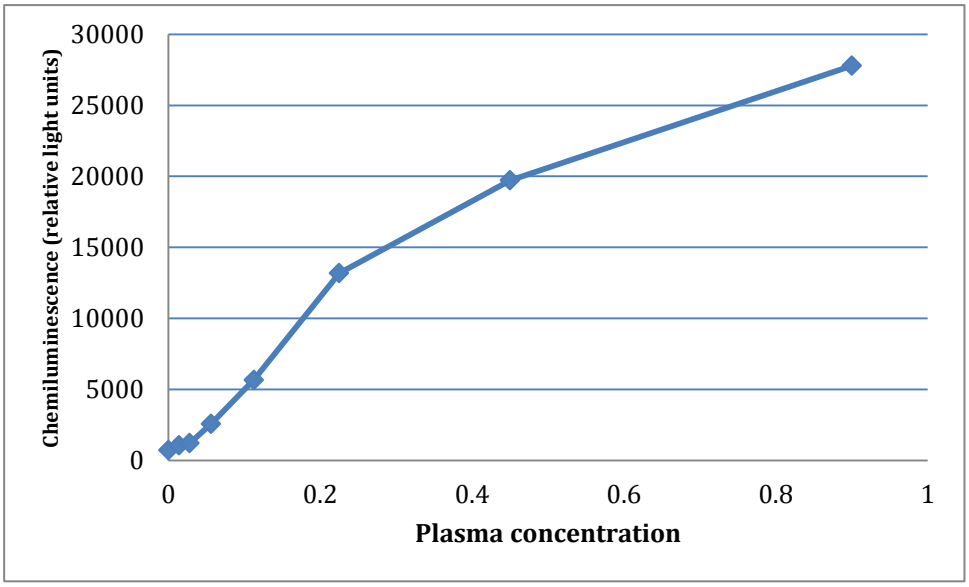


Figure 6.12 Standard curve for the carboxypeptidase B2 immunoassay.

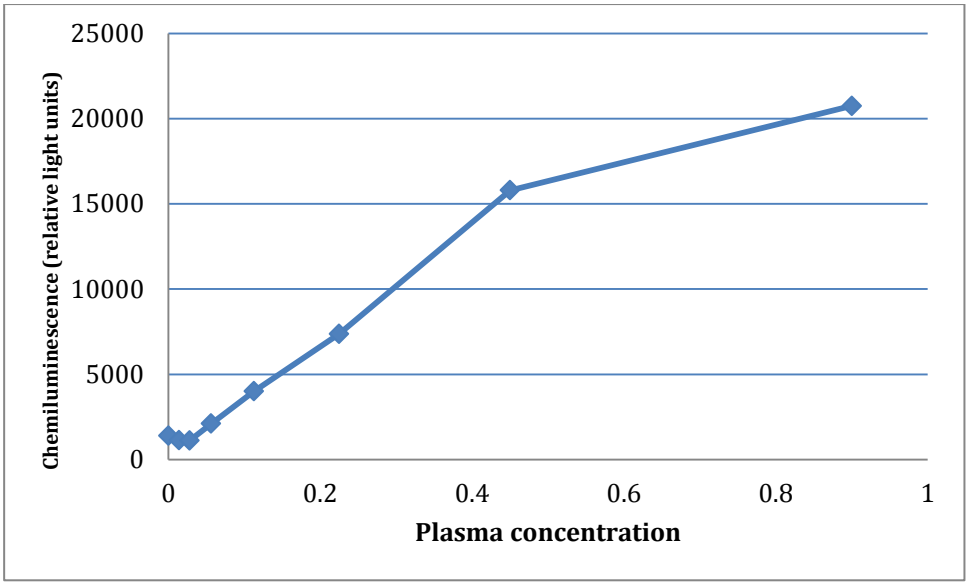


Figure 6.13 Standard curve for the tetranectin immunoassay.

The carboxypeptidase B2 and tetranectin levels for the eighty patient samples (range 7486.90 – 19481.10 RLU) and (range 5428.70 - 19676.90 RLU) respectively, were located on the linear aspect of their respective standard curve. This indicated that the plasma samples were diluted sufficiently to overcome any potential limitations from antibody binding saturation, which would invalidate the results.

There was a statistically significant difference in carboxypeptidase B2 levels between the South Asians patients and the Caucasian patients with CAD (14115.97 RLU ± 2703.03 vs. 12966.49 RLU ± 2332.18; P=0.0451) Figure 6.14. No significant difference in tetranectin levels was observed between the South Asians with CAD and the Caucasian patients with CAD (11736.39 RLU ± 2898.90 vs. 11598.12 ± 2797.01; P=0.829) respectively, Figure 6.15.

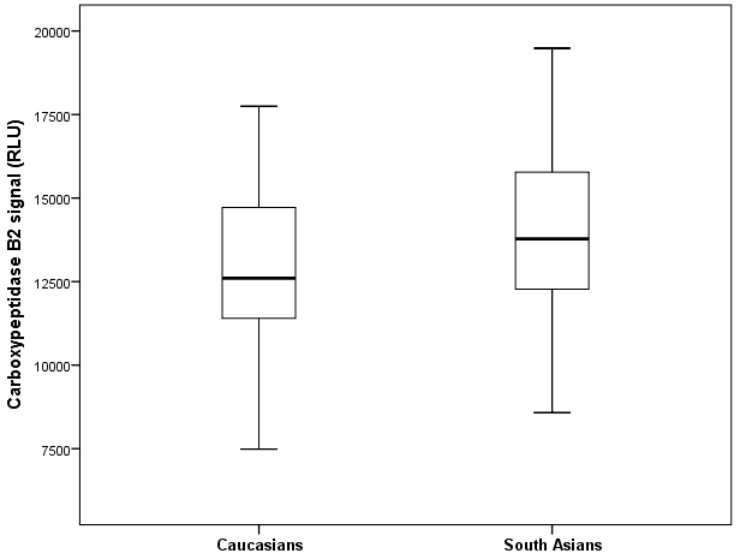


Figure 6.14 Boxplot showing the carboxypeptidase B2 signal (RLU) for the two ethnicities (P=0.045).

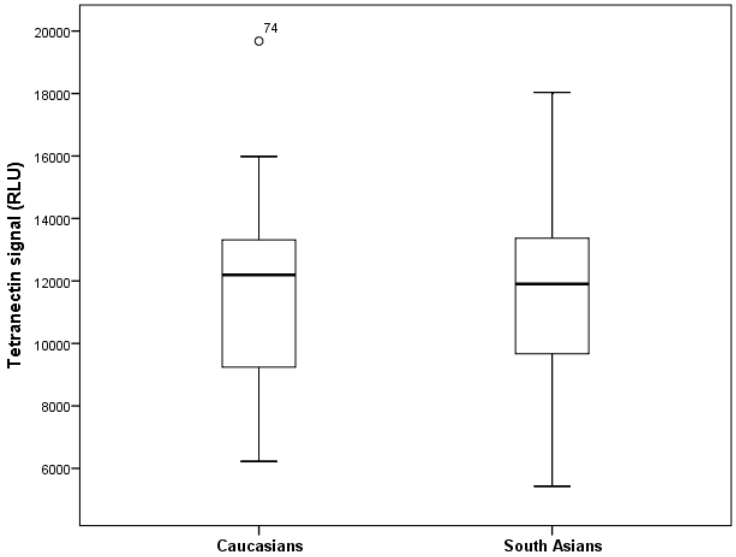


Figure 6.15 Boxplot showing the Tetranectin signal (RLU) for the two ethnicities (P=0.829).

6.9 **Discussion**

The identification of subtle perturbations in protein levels between different ethnicities in the setting of CAD, may further our understanding of this complex process. The use of a highly sophisticated MS platform identified a number of differences in protein intensities between South Asians and Caucasian patients with CAD. Due to time constraints, only 2 of the 28 differentially expressed proteins were taken forward for verification using the clinical plasma samples. Both carboxypeptidase B2 and tetranectin, albeit indirectly, play a role in fibrinolysis. The MS platform revealed an up regulation of carboxypeptidase B2 and a down regulation of tetranectin in the South Asian patients compared to the Caucasian patients, potentially contributing to their pro-thrombotic milieu. Using in-house immunoassays for quantification and verification of the biomarkers, only plasma carboxypeptidase B2 demonstrated a significant difference between the two ethnic cohorts. The lack of difference in tetranectin levels between the two cohorts may be simply related to assay design or due to low power of study from the small sample number, to truly identify any significant difference. In contrast, plasma levels of carboxypeptidase B2 were significantly higher in South Asians patients compared to their Caucasian counterparts, corroborating the MS findings. The differential expression may be a result of gene-environment interactions or gene-gene interactions. Interestingly, a study by Juhan-Vague et al reported that the influence of cardiovascular risk factors on plasma levels of carboxypeptidase B2 is negligible (Juhan-Vague *et al*, 2000). In his primary prevention study, only waist hip ratio, blood pressure and fibrinogen were related with carboxypeptidase B2 levels in males in univariate analysis. In multivariate analysis, only waist hip ratio remained independently predictive of carboxypeptidase B2 levels, with an R^2 of 2% (Juhan-Vague *et al*, 2000). This contrasts with the findings in this study, whereby in a step wise multivariate model, that included conventional cardiovascular risk factors and waist hip ratio, only South Asian ethnicity and LDL-C levels remained independently predictive of carboxypeptidase B2 levels. This final model explained a greater proportion of the variance in carboxypeptidase B2 levels (18.3%). But this highlights that a large proportion of the variance remains unexplained, with Juhan-Vague suggesting the protein levels may be more strongly

influenced by gene-gene interactions, in contrast to the other pro-thrombotic proteins such as PAI-1 and fibrinogen (Juhan-Vague *et al*, 2000).

Following on from this, in a study by Henry et al, seven polymorphisms in the carboxypeptidase B2 gene were found to influence plasma carboxypeptidase B2 levels in Caucasian males without CAD (Henry *et al*, 2001). Furthermore in multivariate analysis, two specific polymorphisms (C+1542G and ala147thr) were independently associated with plasma carboxypetidase B2 levels, accounting for 62% of its variation, reinforcing the strong genetic component (Henry *et al*, 2001). The higher levels in the South Asians may reflect a predominance of certain polymorphisms that determine its plasma level.

The relationship between carboxypeptidase B2 levels and hard clinical outcomes is conflicting. In stable CAD patients, peripheral plasma levels of precursor carboxypeptidase and intracoronary levels of carboxypeptidase B2 were significantly higher in stable CAD patients compared to control subjects (Silveira *et al*, 2000; Schroeder *et al*, 2002). Contrasting with such findings, the PRIME study found no difference in carboxypeptidase B2 levels between French and Irish patients that developed MI or coronary death compared to age matched controls at 5 years. Furthermore, French patients with higher carboxypeptidase levels endured less cardiovascular endpoints, however this association disappeared after adjustment for cardiovascular risk factors (Juhan-Vague, Morange and PRIME Study Group, 2003). In keeping with the initial studies, Tregouet et al reported that the ratio of carboxypeptidase B2 active/inactive (a/i) forms was a surrogate marker of risk in patients with angiographically confirmed CAD (Tregouet *et al*, 2009). CAD patients that endured cardiovascular death had higher carboxypeptidase a/i levels compared to event free survivors, with levels also higher in those patients that went on to develop non-fatal MI compared to the event free survivors. This unique marker of activity was predictive of cardiovascular death even after adjustment for cardiovascular risk factors (Tregouet *et al*, 2009). No association was observed for carboxypeptidase B2 and the cardiovascular outcomes. The lack of observation may be due to differences in assay design, which may obscure a true representation of actual levels. The findings from Tregouet et al reinforce the dogma that carboxypeptidase B2 is

inherently involved in fibrinolysis and its activation impedes clot dissolution, which ultimately culminates in adverse outcomes (Tregouet *et al*, 2009). More recently, using a sensitive immunoassay both intracoronary and peripheral venous sampling of carboxypeptidase B2 were higher in patients with acute MI compared to control subjects (Leenaerts *et al*, 2015). This novel pathway is currently being investigated for therapeutic manipulation with various carboxypeptidase derived peptides shown to modulate its activation and potentially attenuate its pro-thrombotic nature (Plug *et al*, 2015). The higher levels of carboxypeptidase B2 in the South Asians determined by MS and later verified in the clinical samples suggest that this may be accurate for this cohort of subjects, contributing towards a pro-thrombotic tendency. The small sample size limits its generalisability to larger populations. Replication in a larger population is required for a true meaningful interpretation.

One of the limitations of this study is the heterogeneity in the two ethnic groups. Matching for age and comorbidities was difficult between the groups; however, the use of linear regression analysis served to try and control for such differences.

Another limitation is the lack of correction for multiple testing using the Bonferroni correction. Progenesis Qi uses a FDR which alters the p value to account for multiple testing, however this is less stringent than Bonferroni and may potentially identify proteins incorrectly.Due to limitations of time only two candidate biomarkers were chosen for verification, potentially overlooking other proteins that may have clinical significance.

The clinical utility of proteomics is unquestionable, as it facilitates a deep exploration of the plasma proteome to yield novel markers of risk. The use of proteomics in isolation may be short-sighted, as an integrative approach including genomic, transcriptomic, glycomic and metabolomic information will further our understanding of disease pathobiology and enhance risk stratification of patients and help to deliver a new era of personalised medicine.

Chapter 7

7 Conclusion and future direction

CAD is a leading cause of mortality in the UK, with South Asians at increased risk compared to their Caucasian counterparts, partly due to their disproportionately high prevalence of diabetes. Differences exist in their lipoprotein profiles with South Asians tending to have lower HDL and higher triglycerides. The epidemiological evidence linking HDL with cardiovascular risk stems from the Framingham study, however the failure of AIM high study and CETP inhibitors in translating increased HDL to improved survival has questioned its importance. Greater appreciation of the functional capacity of the HDL particle is being acknowledged and paving the way for new research, owing in part to its carriage of a unique subset of low abundant proteins which exert diverse roles. The study of such low abundant proteins using proteomics is challenging, due to the huge dynamic range conferred by the plasma proteome and the limited resolving power offered by MS. In this study a unique novel resin was used for an unbiased lipoprotein pull down, reducing the complexity of the sample processing and enabling MS analysis. Various methodological approaches were tested to accurately assess the lipoproteomic cargo. This resin was shown to be a robust, a reliable and an efficient platform in which to explore the lipoproteins and avoided the potential pitfalls associated with sequential density ultracentrifugation. The combination with high definition ion mobility enabled MS analysis yielded unparalleled data acquisition, thus improving protein discovery.

The remit of this study was to explore the lipoproteomic differences between South Asian patients and Caucasian patients with CAD. The design of this experiment required preliminary workup.

Firstly, lipoproteins are dynamic particles that are susceptible to the acquisition or depletion of certain proteins depending on the clinical state. The cornerstone of treatment for patient with suspected or confirmed CAD is with the prescription of statins. Hence, the role of statin therapy on lipoproteins and their associated protein cargo was investigated in patients with hypercholesterolemia, using this novel resin. Statin therapy was shown to remodel the lipoproteins with an up regulation of proteins concerned with cytoskeletal structure, antioxidation and

antiproliferative properties. Unexpectedly, a novel adipokine called adipsin was found to be down regulated with statin therapy. This was an interesting discovery, as reduced levels would predispose to diabetes and potentially explain the controversial link between statin use and new onset diabetes (Ridker *et al*, 2012; Sattar *et al*, 2010). However, verification of this proved unsuccessful in the clinical samples. This sub study highlighted that statins mediate both lipid dependent and lipid independent effects, the latter of which may be due to disturbances in protein expression.

Secondly, the lipoproteomic differences were explored between CAD patients and control subjects matched for statins use, to negate its effect, using a similar lipoproteomic discovery workflow. Of the two hundred and seventy two proteins identified between the two cohorts, 168 demonstrated significant differential expression. Due to the vast differences, discriminant analysis was performed to identify the fewest number of proteins that would accurately predict group membership. The final model contained seven proteins, which classified all patients/controls correctly.

Finally, the natural progression of this work was to then explore the lipoproteomic differences between South Asian patients and Caucasian patients with CAD. Patients were recruited 3 months after their acute event and a minimum of 1 month of statin stabilisation, to avoid any bias from acute inflammation and from fluxes in lipoprotein levels respectively. The strengths of this study were the large number of biological cases (100 patients) explored, as typically most proteomic studies investigate 10 vs. 10 and the use of high definition MS analysis. Using the reproducible lipoproteomic discovery pipeline, 272 proteins were identified with 28 proteins demonstrating significant differential expression between the South Asian cohort and the Caucasian cohort. Proteins concerned with inflammation, coagulation, redox reactions, extracellular remodelling and obesity were found to be dysregulated between the two ethnicities. Two interesting proteins carboxypeptidase B2 and tetranectin were significantly up regulated and down regulated in the South Asians patients respectively, contributing towards a pro-thrombotic state. In verification studies the association for higher levels of carboxypeptidase B2 persisted in the South Asians compared with the Caucasian

patients with CAD, lending to strength to the discovery workflow. The higher levels of carboxypeptidase B2 in the South Asians may be due to an inherent genetic predisposition, but requires further replication and validation in a larger cohort of patients for any meaningful interpretation to be made. The notion of lipoproteins as simplistic particles, shuttling lipid around the body is slowly evolving to encompass an integral role in inflammation and coagulation. Their ability to travel in plasma and their extrusion into the sub endothelial space for participation in atherosclerosis provides a suitable platform in which to study this complex disease. The role of lipoproteomics as highlighted by this study is important, but must be used in conjunction with other "omics" based approaches for a complete understanding of this condition, especially for biomarker discovery.

The future of this work lies in the ability to reproduce the results in a larger cohort of patients. The exploration of the lipoprotein associated protein cargoes using this novel resin is not limited to CAD, but may be used in other cardiovascular diseases. The interesting link between statin use and lower adipsin levels requires further exploration with a possible targeted approach.

The alteration of carboxypeptidase B2 in the South Asian patients requires validation in a large cohort of patients. Another potential direction is to analyse the data specifically searching for post translational changes such oxidative markers, that may be differentially expressed between the different groups and potentially confer adverse risk.

The use of proteomics to determine protein function in biological specimens is currently hampered by various technical challenges. The biological tissue selected for proteomic exploration may hinder full exploration, as for example the dynamic range for plasma is well beyond the scope of covered by conventional bench top MS. Hence a number of depletion steps are required to bring the dynamic range of the sample to within the capability of the MS, with the potential of losing interesting proteins in the depletome. In this study the lipoproteome was chosen for its relative simplicity in comparison to that conferred by the plasma proteome. In this study, the samples were subjected bottom-up approach with a number of offline processing steps such as lipo-affinity co-incubation, washing, reduction,

alkylation and digestion prior to MS analysis. Such processing was significantly time consuming, with errors only being identified after MS analysis. A possible way of overcoming this bottleneck would be to combine the pre-processing stages with online MS analysis to improve through-put. Conversely, greater affordability of top-down MS platforms may certainly streamline this stage. In this study HDMS^{e} ion mobility enabled data acquisition was performed to improve protein coverage. However further improvements in MS resolution are required for a deeper understanding of the plasma proteome. The data generated from this study was staggering, ranging up to 6 terabytes. The software used for bioinformatic data analysis was not specifically designed for such large cohort studies. The expression analysis for the ethnicity study using progenesis Qi consumed almost 3months. Although the computers used for this analysis were high performance machines with 60GB of RAM, more sophisticated supercomputers should have been utilised for rapid data analysis.

The field of proteomic is evolving, with required advancements in sample preparation, MS analysis and data processing, for it to be at the forefront of biomarker discovery.

Appendicies

8 Appendicies

8.1 Appendix A

Ethical Approval:

NRES Committee East Midlands - Northampton
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Nottingham
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Telephone: 0115 8839435
Facsimile: 0115 8839294

28 March 2013

Prof Leong Ng
Professor of Medicine & Therapeutics
Universiy of Leicester,
Cardiovascular Sciences
Level 4 Clinical Science Building
Leicester Royal Infirmary
LE2 7LX

Dear Prof Ng

Study title:	**The Impact of Ethnicity on the High Density Lipoprotein (HDL) proteome**
REC reference:	**13/EM/0049**
IRAS project ID:	**114422**

Thank you for your letter of 20 March 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Miss Georgia Copeland , NRESCommittee.EastMidlands-Northampton@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of insurance or indemnity		07 January 2013
Investigator CV		08 January 2013
Other: CV - Dr Bhandari		08 January 2013
Other: Recruitment of Healthy Volunteers Poster	1	08 January 2013
Participant Consent Form: Control	2	10 March 2013
Participant Consent Form: Patient	2	10 March 2013
Participant Information Sheet: Control	2	10 March 2013
Participant Information Sheet: Patient	2	10 March 2013
Protocol	1	01 November 2012
REC application	114422/400849/1/41	08 January 2013
Response to Request for Further Information		20 March 2013

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/EM/0049	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Mr John Aldridge
Chair

Email:NRESCommittee.EastMidlands-Northampton@nhs.net

Enclosures: After ethical review – guidance for researchers

8.2 **Appendix B**

Total list of all proteins identified using LRA.

Accession number	Description
Q9NR81-2;Q9NR81-3;Q9NR81-4	Isoform 2 of Rho guanine nucleotide exchange factor 3
P53990;P53990-2;P53990-4;P53990-5	IST1 homolog
Q9BQ52;Q9BQ52-2;Q9BQ52-4	Zinc phosphodiesterase ELAC protein 2
P49407-2;P49407	Isoform 1B of Beta-arrestin-1
O60304	Zinc finger protein 500
Q9P2N6;Q9P2N6-2;Q9P2N6-3;Q9P2N6-4;Q9P2N6-5;Q9P2N6-6;Q9P2N6-8	KAT8 regulatory NSL complex subunit 3
P20742;P20742-2	Pregnancy zone protein
P18428	Lipopolysaccharide-binding protein
A6NCW7;P0C7I0	Inactive ubiquitin carboxyl-terminal hydrolase 17-like protein 4
Q01449	Myosin regulatory light chain 2, atrial isoform
Q7L576	Cytoplasmic FMR1-interacting protein 1
P68366	Tubulin alpha-4A chain
P43627	Killer cell immunoglobulin-like receptor 2DL2
Q86VG3	Uncharacterized protein C11orf74
Q9P2D7;Q9P2D7-2;Q9P2D7-4	Dynein heavy chain 1, axonemal
Q8NHU2;Q02218-2;Q8NHU2-3;Q8NHU2-4	Uncharacterized protein C20orf26
P46063	ATP-dependent DNA helicase Q1
Q8N335	Glycerol-3-phosphate dehydrogenase 1-like protein
Q63HN8;Q63HN8-4	E3 ubiquitin-protein ligase RNF213
Q86TU6	Putative uncharacterized protein encoded by LINC00523
Q13561;Q13561-2;Q13561-3	Dynactin subunit 2
Q8N323	NXPE family member 1
Q9NRE2;Q9NRE2-2	Teashirt homolog 2
Q7Z569	BRCA1-associated protein
Q16635-6;Q16635;Q16635-2;Q16635-3;Q16635-4;Q16635-5;Q16635-7;Q16635-8;Q16635-9	Isoform 6 of Tafazzin
A9UHW6-2;A9UHW6;A9UHW6-3	Isoform 2 of MIF4G domain-containing protein
Q92667;Q92667-2	A-kinase anchor protein 1, mitochondrial
Q13127-4;Q13127;Q13127-2;Q13127-3	Isoform 4 of RE1-silencing transcription factor
Q86XP1;Q86XP1-2;Q86XP1-3;Q86XP1-5	Diacylglycerol kinase eta
O75400;O75400-2;O75400-3	Pre-mRNA-processing factor 40 homolog A
Q96PL1	Secretoglobin family 3A member 2
Q9Y5R5;Q9Y5R5-3	Doublesex- and mab-3-related transcription factor 2
Q16526;Q49AN0;Q49AN0-2	Cryptochrome-1
P04921-2	Isoform Glycophorin-D of Glycophorin-C
Q4JDL3;Q4JDL3-3;Q4JDL3-4;Q4JDL3-7	Tyrosine-protein phosphatase non-receptor type 20
Q7L0Q8-2	Isoform 2 of Rho-related GTP-binding protein RhoU
Q8NG06	Tripartite motif-containing protein 58
Q8IVF5-5;Q8IVF5;Q8IVF5-2	Isoform 5 of T-lymphoma invasion and metastasis-inducing protein 2
P01598	Ig kappa chain V-I region EU
P14618;P14618-2;P14618-3	Pyruvate kinase PKM

Q9BPY3	Protein FAM118B
Q8WU03	Glycine N-acyltransferase-like protein 2
Q96NL6;Q96NL6-2;Q96NL6-3	Sodium channel and clathrin linker 1
Q8IZU1	Protein FAM9A
Q7L5D6;Q7L5D6-2	Golgi to ER traffic protein 4 homolog
P10275	Androgen receptor
Q8NBT3	Transmembrane protein 145
Q08257;Q08257-2;Q08257-3	Quinone oxidoreductase
A9Z1Z3	Fer-1-like protein 4
Q9P242	Neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adapter 2
Q96NH3-4;Q96NH3;Q96NH3-5	Isoform 2 of Protein broad-minded
Q0P6D2;Q0P6D2-2	Protein FAM69C
Q99542-3	Isoform 2 of Matrix metalloproteinase-19
O43815;O43815-2	Striatin
Q8WWZ7	ATP-binding cassette sub-family A member 5
Q9P289;Q9P289-3	Serine/threonine-protein kinase MST4
P01344;P01344-2;P01344-3	Insulin-like growth factor II
Q9P283;Q9P283-2;Q9P283-3;Q9P283-4	Semaphorin-5B
Q9BXY5;Q9BXY5-2;Q9BXY5-3	Calcyphosin-2
Q8NI60-2	Isoform 2 of Chaperone activity of bc1 complex-like, mitochondrial
Q9BWP8-4;Q9BWP8;Q9BWP8-10;Q9BWP8-2;Q9BWP8-3;Q9BWP8-5;Q9BWP8-6;Q9BWP8-7;Q9BWP8-8;Q9BWP8-9	Isoform 4 of Collectin-11
O60344;O60344-4	Endothelin-converting enzyme 2
O14830;O14830-2	Serine/threonine-protein phosphatase with EF-hands 2
Q8NFI3;Q8NFI3-2;Q8NFI3-3	Cytosolic endo-beta-N-acetylglucosaminidase
P08575-2;P08575	Isoform 2 of Receptor-type tyrosine-protein phosphatase C
P02545-3	Isoform ADelta10 of Prelamin-A/C
P00746	Complement factor D
Q7Z7G2	Complexin-4
P19784	Casein kinase II subunit alpha'
Q8TCE6;Q6NSW5;Q8TCE6-2	Protein FAM45A
Q9Y620	DNA repair and recombination protein RAD54B
Q86W92;Q86W92-2;Q86W92-4;Q86W92-5	Liprin-beta-1
Q49AM1	mTERF domain-containing protein 3, mitochondrial
Q8TC92	Ecto-NOX disulfide-thiol exchanger 1
Q99962;Q99961;Q99961-2	Endophilin-A1
Q6ZNA1	Zinc finger protein 836
P07996	Thrombospondin-1
P55058-3	Isoform 3 of Phospholipid transfer protein
O43715	TP53-regulated inhibitor of apoptosis 1
P15428;P15428-5	15-hydroxyprostaglandin dehydrogenase [NAD(+)]
O95445-2	Isoform 2 of Apolipoprotein M
Q8NFW5-2	Isoform 2 of Diencephalon/mesencephalon homeobox protein 1
Q9Y6K1;Q9Y6K1-3	DNA (cytosine-5)-methyltransferase 3A
Q9H668	CST complex subunit STN1
Q8TAX9-2;Q8TAX9;Q8TAX9-3;Q8TAX9-4;Q8TAX9-6	Isoform 2 of Gasdermin-B
P04434	Ig kappa chain V-III region VH (Fragment)

P22352	Glutathione peroxidase 3
P0DJI8	Serum amyloid A-1 protein
Q86YS6;A6NDJ8	Ras-related protein Rab-43
Q5TCS8;Q5TCS8-5;Q5TCS8-6	Adenylate kinase 9
P08758	Annexin A5
P11226	Mannose-binding protein C
Q92616	Translational activator GCN1
Q7L804	Rab11 family-interacting protein 2
Q2TV78;Q2TV78-2	Putative macrophage stimulating 1-like protein
P32856-3;P32856;P32856-2	Isoform 2 of Syntaxin-2
P07360	Complement component C8 gamma chain
Q9NR50-3;Q9NR50;Q9NR50-2	Isoform 3 of Translation initiation factor eIF-2B subunit gamma
Q5VSL9	Striatin-interacting protein 1
P0CB38	Polyadenylate-binding protein 4-like
Q9UBF8;Q9UBF8-2;Q9UBF8-3	Phosphatidylinositol 4-kinase beta
Q658N2	WSC domain-containing protein 1
O75128-2;O75128;O75128-3;O75128-7	Isoform 2 of Protein cordon-bleu
P41222	Prostaglandin-H2 D-isomerase
Q8WVJ2	NudC domain-containing protein 2
Q96RR4-6;Q96RR4;Q96RR4-2;Q96RR4-3;Q96RR4-4;Q96RR4-5;Q96RR4-7	Isoform 6 of Calcium/calmodulin-dependent protein kinase kinase 2
Q9BU19;Q9BU19-2;Q9BU19-5	Zinc finger protein 692
O15305	Phosphomannomutase 2
Q5K130	Putative chronic lymphocytic leukemia up-regulated protein 1 opposite strand transcript protein
Q7Z5L7;Q7Z5L7-2;Q7Z5L7-3;Q7Z5L7-4	Podocan
P02766	Transthyretin
Q15751	Probable E3 ubiquitin-protein ligase HERC1
O95389;O95389-2	WNT1-inducible-signaling pathway protein 3
Q9BW83;Q9BW83-2	Intraflagellar transport protein 27 homolog
Q86UP8;Q6EKJ0;Q6EKJ0-2;Q86UP8-2;Q86UP8-3;Q86UP8-5	General transcription factor II-I repeat domain-containing protein 2A
Q96FT9;Q96FT9-2;Q96FT9-3	Intraflagellar transport protein 43 homolog
P01767	Ig heavy chain V-III region BUT
Q01432-5;Q01432;Q01432-2;Q01432-3;Q01432-4	Isoform 3 of AMP deaminase 3
P02549;P02549-2	Spectrin alpha chain, erythrocytic 1
O43741	5'-AMP-activated protein kinase subunit beta-2
Q7L5N7	Lysophosphatidylcholine acyltransferase 2
Q9NP73;Q9NP73-3	Putative bifunctional UDP-N-acetylglucosamine transferase and deubiquitinase ALG13
Q15746-2;Q15746;Q15746-3;Q15746-4;Q15746-5;Q15746-6;Q15746-7	Isoform 2 of Myosin light chain kinase, smooth muscle
Q5TD97	Four and a half LIM domains protein 5
P11532-4;P11532;P11532-2;P11532-3	Isoform 3 of Dystrophin
Q659C4;Q659C4-2;Q659C4-5;Q659C4-6;Q659C4-7;Q659C4-9	La-related protein 1B
Q6DN12-2;Q6DN12;Q6DN12-6	Isoform 2 of Multiple C2 and transmembrane domain-containing protein 2

Q8NDV7-6;Q8NDV7;Q8NDV7-2;Q8NDV7-5	Isoform 6 of Trinucleotide repeat-containing gene 6A protein
P01717	Ig lambda chain V-IV region Hil
Q8WWZ4;Q8WWZ4-2	ATP-binding cassette sub-family A member 10
P04207	Ig kappa chain V-III region CLL
O60271-3;O60271;O60271-2;O60271-4;O60271-5	Isoform 3 of C-Jun-amino-terminal kinase-interacting protein 4
Q8N2C7;Q8N2C7-4	Protein unc-80 homolog
Q6P5Q4	Leiomodin-2
Q9NRZ9-6;Q9NRZ9;Q9NRZ9-2;Q9NRZ9-3;Q9NRZ9-4;Q9NRZ9-5	Isoform 6 of Lymphoid-specific helicase
O75533	Splicing factor 3B subunit 1
O95785;O95785-2	Protein Wiz
Q8WXH2;Q8WXH2-2	Junctophilin-3
Q05195-2;Q05195	Isoform 2 of Max dimerization protein 1
Q13642;Q13642-1;Q13642-3;Q13642-4;Q13642-5	Four and a half LIM domains protein 1
Q9Y272	Dexamethasone-induced Ras-related protein 1
Q5VU43;Q5VU43-3;Q5VU43-4	Myomegalin
Q92623	Tetratricopeptide repeat protein 9A
Q9NP58-4;Q9NP58	Isoform 2 of ATP-binding cassette sub-family B member 6, mitochondrial
O15294-3;O15294;O15294-2;O15294-4	Isoform 1 of UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit
Q8N8B7-2;Q8N8B7	Isoform 2 of Transcription elongation factor A N-terminal and central domain-containing protein
Q9UBC2;Q9UBC2-2	Epidermal growth factor receptor substrate 15-like 1
Q7LBC6;Q7LBC6-2;Q7LBC6-3	Lysine-specific demethylase 3B
P01624	Ig kappa chain V-III region POM
P48751-3;P48751;P48751-2	Isoform 3 of Anion exchange protein 3
Q96Q89-4;Q96Q89;Q96Q89-2;Q96Q89-3	Isoform 4 of Kinesin-like protein KIF20B
Q96BT7;Q96BT7-2;Q96BT7-3;Q96BT7-4	Alkylated DNA repair protein alkB homolog 8
Q9UKN8	General transcription factor 3C polypeptide 4
Q9P0W5-6;Q9P0W5;Q9P0W5-2;Q9P0W5-3;Q9P0W5-4;Q9P0W5-5	Isoform 6 of Schwannomin-interacting protein 1
P27816-6;P27816;P27816-2;P27816-5	Isoform 6 of Microtubule-associated protein 4
Q03001-13;Q03001-12	Isoform 8 of Dystonin
P06310	Ig kappa chain V-II region RPMI 6410
O00295	Tubby-related protein 2
Q14432	cGMP-inhibited 3',5'-cyclic phosphodiesterase A
P14927-2	Isoform 2 of Cytochrome b-c1 complex subunit 7
Q8WXS3;Q8WXS3-3	Brain and acute leukemia cytoplasmic protein
P01764;P01771	Ig heavy chain V-III region VH26
P55201;P55201-2;P55201-3;P55201-4	Peregrin
Q10588	ADP-ribosyl cyclase 2
A0PJZ0	Putative ankyrin repeat domain-containing protein 20A5
P57796;P57796-2	Calcium-binding protein 4
P02776	Platelet factor 4
Q9H5V9;Q9H5V9-2;Q9H5V9-3	UPF0428 protein CXorf56
P39060-1;P39060;P39060-2	Isoform 2 of Collagen alpha-1(XVIII) chain
P14151;P14151-2	L-selectin

Q86XL3;Q86XL3-3	Ankyrin repeat and LEM domain-containing protein 2
Q9ULK4-4;Q9ULK4;Q9ULK4-2;Q9ULK4-3;Q9ULK4-5;Q9ULK4-6	Isoform 4 of Mediator of RNA polymerase II transcription subunit 23
P06733;P06733-2	Alpha-enolase
P31327;P31327-2;P31327-3	Carbamoyl-phosphate synthase [ammonia], mitochondrial
P05534	HLA class I histocompatibility antigen, A-24 alpha chain
P33151	Cadherin-5
O14717;O14717-2;O14717-3;O14717-5;O14717-6	tRNA (cytosine(38)-C(5))-methyltransferase
Q9UPQ7	E3 ubiquitin-protein ligase PDZRN3
Q00987-11;Q00987;Q00987-10;Q00987-2;Q00987-3;Q00987-5;Q00987-8;Q00987-9	Isoform 11 of E3 ubiquitin-protein ligase Mdm2
Q9H175	Cysteine/serine-rich nuclear protein 2
Q969H4;Q969H4-2	Connector enhancer of kinase suppressor of ras 1
P49908	Selenoprotein P
Q8NHM5-4;Q8NHM5;Q8NHM5-2	Isoform 4 of Lysine-specific demethylase 2B
Q8IX03	Protein KIBRA
O43361	Zinc finger protein 749
Q6ZQQ6;Q6ZQQ6-2	WD repeat-containing protein 87
Q86XE0;Q86XE0-2	Sorting nexin-32
Q2M3G0-4;Q2M3G0;Q2M3G0-2;Q2M3G0-3	Isoform 4 of ATP-binding cassette sub-family B member 5
O14524-2;O14524	Isoform 2 of Transmembrane protein 194A
P12104	Fatty acid-binding protein, intestinal
Q659A1;Q659A1-2	NMDA receptor-regulated protein 2
P49321;P49321-3;P49321-4	Nuclear autoantigenic sperm protein
A6PVI3	Nuclear cap-binding protein subunit 2-like
Q7L5N1	COP9 signalosome complex subunit 6
P10451-4;P10451;P10451-2;P10451-3;P10451-5	Isoform D of Osteopontin
Q9NZT1	Calmodulin-like protein 5
Q13242	Serine/arginine-rich splicing factor 9
Q9NZU7;Q9NZU7-1;Q9NZU7-2;Q9NZU7-3	Calcium-binding protein 1
Q96MP5	Zinc finger SWIM domain-containing protein 3
P01714;P06318;P06319	Ig lambda chain V-III region SH
Q6NXT1	Ankyrin repeat domain-containing protein 54
P04206	Ig kappa chain V-III region GOL
E7ERA6	RING finger protein 223
Q96S66;Q96S66-2;Q96S66-3;Q96S66-4	Chloride channel CLIC-like protein 1
Q6P4F7	Rho GTPase-activating protein 11A
P53671	LIM domain kinase 2
Q6I9Y2	THO complex subunit 7 homolog
P04278;P04278-2;P04278-3;P04278-4	Sex hormone-binding globulin
O15492	Regulator of G-protein signaling 16
P23528	Cofilin-1
Q96TC7;Q96TC7-2	Regulator of microtubule dynamics protein 3
Q3MII6;Q3MII6-2	TBC1 domain family member 25
Q155Q3;Q155Q3-2	Dixin
P01703	Ig lambda chain V-I region NEWM
Q5VTT2	Uncharacterized protein C9orf135

Q00G26	Perilipin-5
Q8WW24	Tektin-4
P35243	Recoverin
Q9BZJ0;Q9BZJ0-3;Q9BZJ0-4;Q9BZJ0-5	Crooked neck-like protein 1
P30447	HLA class I histocompatibility antigen, A-23 alpha chain
P53667;P53667-2;P53667-4	LIM domain kinase 1
Q5R372;Q5R372-3;Q5R372-4;Q5R372-9	Rab GTPase-activating protein 1-like
P04209	Ig lambda chain V-II region NIG-84
Q8N7W2;Q8N7W2-3	BEN domain-containing protein 7
A7E2Y1	Myosin-7B
Q86XD8	AN1-type zinc finger protein 4
Q9NR09	Baculoviral IAP repeat-containing protein 6
Q9BUA6	Myosin regulatory light chain 10
Q12774	Rho guanine nucleotide exchange factor 5
Q8NCI6	Beta-galactosidase-1-like protein 3
Q8NCI6-2	Isoform 2 of Beta-galactosidase-1-like protein 3
P08123	Collagen alpha-2(I) chain
Q5T5X7	BEN domain-containing protein 3
Q01064;Q01064-2	Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B
Q86WT6	E3 ubiquitin-protein ligase TRIM69
P08133;P08133-2	Annexin A6
Q9BYJ4-2;Q9BYJ4	Isoform 2 of Tripartite motif-containing protein 34
C9J1S8	Tripartite motif-containing protein 49D1
Q9P217	Zinc finger SWIM domain-containing protein 5
P07737	Profilin-1
Q8IYS0;O43680;Q8IYS0-2	GRAM domain-containing protein 1C
P04180	Phosphatidylcholine-sterol acyltransferase
O43247	Testis-expressed sequence 33 protein
Q8N945-3;Q8N945;Q8N945-2	Isoform 3 of PRELI domain-containing protein 2
Q8IYW2	Tetratricopeptide repeat protein 40
P01778	Ig heavy chain V-III region ZAP
P41134-2	Isoform ID-B of DNA-binding protein inhibitor ID-1
Q9BXN6;Q8TAD1	Sperm protein associated with the nucleus on the X chromosome D
Q96JG9	Zinc finger protein 469
P29622	Kallistatin
P06681;P06681-3	Complement C2
P01042	Kininogen-1
Q9HCH0;Q9HCH0-2;Q9HCH0-3	Nck-associated protein 5-like
Q1ED39	Lysine-rich nucleolar protein 1
P43628	Killer cell immunoglobulin-like receptor 2DL3
P02786	Transferrin receptor protein 1
P12259	Coagulation factor V
P02748	Complement component C9
A1XBS5-3	Isoform 3 of Protein FAM92A1
P02774-3;P02774	Isoform 3 of Vitamin D-binding protein
Q14320	Protein FAM50A
A8MWC7	Putative neugrin-like protein

Q15413;Q15413-2;Q15413-3	Ryanodine receptor 3
P35579;P35579-2	Myosin-9
O43829	Zinc finger and BTB domain-containing protein 14
P48740;P48740-3	Mannan-binding lectin serine protease 1
Q86X27-2	Isoform 2 of Ras-specific guanine nucleotide-releasing factor RalGPS2
Q03591	Complement factor H-related protein 1
Q02224;Q02224-2	Centromere-associated protein E
Q6P158;Q6P158-2;Q6P158-3	Putative ATP-dependent RNA helicase DHX57
P12524	Protein L-Myc
P13671	Complement component C6
Q92954;Q92954-2;Q92954-5	Proteoglycan 4
Q92934	Bcl2 antagonist of cell death
P00751	Complement factor B
Q9UM54;Q9UM54-1;Q9UM54-2;Q9UM54-4;Q9UM54-5;Q9UM54-6	Unconventional myosin-VI
Q6IC98	GRAM domain-containing protein 4
Q9P2M7-2	Isoform 2 of Cingulin
P30281-4;P30281;P30281-2;P30281-3	Isoform 4 of G1/S-specific cyclin-D3
Q5CZC0;Q5CZC0-2	Fibrous sheath-interacting protein 2
Q86VH2-2	Isoform 2 of Kinesin-like protein KIF27
A5A3E0	POTE ankyrin domain family member F
P02790	Hemopexin
Q99735	Microsomal glutathione S-transferase 2
P09493-10;P07951;P07951-2;P09493;P09493-2;P09493-3;P09493-4;P09493-5;P09493-6;P09493-7;P09493-8;P09493-9	Isoform 10 of Tropomyosin alpha-1 chain
P32320	Cytidine deaminase
Q96NZ9-2;Q96NZ9;Q96NZ9-4	Isoform 2 of Proline-rich acidic protein 1
Q92954-6	Isoform F of Proteoglycan 4
Q6S8J3;A6NI47;Q6S545;Q6S545-2;Q6S5H5;Q6S5H5-2;Q6S5H5-4;Q6S8J3-2;Q6S8J3-3	POTE ankyrin domain family member E
Q92954-3;Q92954-4	Isoform C of Proteoglycan 4
Q96PB7;Q96PB7-2;Q96PB7-3;Q96PB7-5	Noelin-3
A4D161;A4D161-2;A4D161-3	Protein FAM221A
P23142-3	Isoform B of Fibulin-1
P03951;P03951-2	Coagulation factor XI
P16471-3	Isoform 3 of Prolactin receptor
Q99698;Q99698-2	Lysosomal-trafficking regulator
P23142;P23142-2	Fibulin-1
P09871	Complement C1s subcomponent
P01860;Q8TAB3;Q8TAB3-2	Ig gamma-3 chain C region
P40925-2;P40925;P40925-3	Isoform 2 of Malate dehydrogenase, cytoplasmic
Q03164;Q03164-2;Q03164-3	Histone-lysine N-methyltransferase 2A
Q8TDQ1;Q8TDQ1-6	CMRF35-like molecule 1
P00736;Q16653;Q16653-10;Q16653-11;Q16653-2;Q16653-3;Q16653-5;Q16653-6;Q16653-7;Q16653-8;Q16653-9	Complement C1r subcomponent
Q9H799	Uncharacterized protein C5orf42

Q9H799-5	Isoform 2 of Uncharacterized protein C5orf42
Q96BY6;Q96BY6-2;Q96BY6-3	Dedicator of cytokinesis protein 10
P00330	Alcohol dehydrogenase 1
P42356;P42356-2	Phosphatidylinositol 4-kinase alpha
P07357	Complement component C8 alpha chain
Q8TBM7-3	Isoform 3 of Transmembrane protein 254
Q5VU13	V-set and immunoglobulin domain-containing protein 8
Q8IXQ8;Q8IXQ8-2	PDZ domain-containing protein 9
Q9UKF7;Q9UKF7-2	Cytoplasmic phosphatidylinositol transfer protein 1
Q9NWM8	Peptidyl-prolyl cis-trans isomerase FKBP14
Q5JTJ3	Cytochrome c oxidase assembly factor 6 homolog
Q96RN1;Q96RN1-2	Testis anion transporter 1
Q6MZQ0;Q6MZQ0-2;Q6MZQ0-3	Proline-rich protein 5-like
O15078;O15078-2	Centrosomal protein of 290 kDa
P00740	Coagulation factor IX
Q6ZTU2-2;Q6ZTU2;Q6ZTU2-4;Q6ZTU2-5;Q6ZTU2-6	Isoform 2 of EP400 N-terminal-like protein
P19526	Galactoside 2-alpha-L-fucosyltransferase 1
Q9BYG8	Gasdermin-C
P00750;P00750-4	Tissue-type plasminogen activator
P01859	Ig gamma-2 chain C region
Q6P1K2-3	Isoform 3 of Polyamine-modulated factor 1
Q8N6C7	Putative uncharacterized protein encoded by MIR7-3HG
P01857;Q8NE28-2;Q8NE28-3	Ig gamma-1 chain C region
Q96M86	Dynein heavy chain domain-containing protein 1
P19827;P19827-2;P19827-3	Inter-alpha-trypsin inhibitor heavy chain H1
P01008	Antithrombin-III
P04004	Vitronectin
Q6UW02	Cytochrome P450 20A1
P50221-3	Isoform 3 of Homeobox protein MOX-1
O94901-8;O94901;O94901-2;O94901-3;O94901-4;O94901-6;O94901-7;O94901-9	Isoform 8 of SUN domain-containing protein 1
Q6P047	Uncharacterized protein C8orf74
O43543	DNA repair protein XRCC2
Q8NF91-7	Isoform 7 of Nesprin-1
Q16610;Q16610-2;Q16610-4	Extracellular matrix protein 1
P02671;A1A5D9-2;A2A3K4;A2A3K4-2;A6NCD1;O43924;O75311;O75311-2;P02671-2;P23415;P23415-2;P49821;P49821-2;P53671-2;P53671-3;Q13418;Q15070;Q494R4;Q4U2R6;Q5JVL4;Q5JVL4-3;Q5SQS8;Q6PF05;Q6PF05-3;Q70J99;Q70J99-3;Q86TB9;Q86TB9-2;Q86TB9-3;Q86TB9-4;Q8TB03-3;Q8TEX9;Q8TEX9-2;Q92737;Q96FV2;Q96FV2-2;Q99607;Q9BSJ1;Q9BSJ1-2;Q9NX09;Q9NYY3;Q9UH62;Q9ULI2;Q9ULI2-2	Fibrinogen alpha chain
Q9HC23;Q9HC23-2	Prokineticin-2
Q8IYS1	Peptidase M20 domain-containing protein 2
P36980;P36980-2	Complement factor H-related protein 2

O75636	Ficolin-3
Q03468	DNA excision repair protein ERCC-6
Q9Y4E8;Q9Y4E8-2;Q9Y4E8-3	Ubiquitin carboxyl-terminal hydrolase 15
P05154	Plasma serine protease inhibitor
P05090	Apolipoprotein D
Q3SYF9	Keratin-associated protein 19-7
O14529	Homeobox protein cut-like 2
Q01113-3	Isoform 3 of Interleukin-9 receptor
P02751;P02751-11;P02751-14;P02751-15;P02751-16;P02751-17;P02751-3;P02751-4;P02751-6;P02751-7;P02751-8;P02751-9	Fibronectin
Q9BS40	Latexin
P02753	Retinol-binding protein 4
Q8TC17;Q13588;Q8TC17-2	GRB2-related adapter protein-like
Q6ZV56;Q6ZV56-2;Q6ZV56-3	Uncharacterized protein C22orf34
Q8NB25;Q8NB25-2;Q8NB25-3;Q8NB25-4	Protein FAM184A
Q9Y3E7;Q9Y3E7-2	Charged multivesicular body protein 3
Q7Z5P4-2;Q7Z5P4	Isoform 1 of 17-beta-hydroxysteroid dehydrogenase 13
Q01082;Q01082-2;Q01082-3	Spectrin beta chain, non-erythrocytic 1
P02746	Complement C1q subcomponent subunit B
P02656	Apolipoprotein C-III
O75636-2	Isoform 2 of Ficolin-3
Q11206-7;Q11206;Q11206-2;Q11206-3;Q11206-4;Q11206-5;Q11206-6	Isoform 7 of CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 4
Q9ULB4	Cadherin-9
Q99996;P25208;Q99996-2;Q99996-3;Q99996-5;Q99996-6	A-kinase anchor protein 9
Q96KN2	Beta-Ala-His dipeptidase
Q9BXW4	Microtubule-associated proteins 1A/1B light chain 3C
P04114;P49862-2;Q16787-3;Q76EJ3;Q76EJ3-2	Apolipoprotein B-100
Q6P1K2-5	Isoform 5 of Polyamine-modulated factor 1
P28356	Homeobox protein Hox-D9
Q96T52-2	Isoform 2 of Mitochondrial inner membrane protease subunit 2
P01880;P01880-2	Ig delta chain C region
Q8TDR2	Serine/threonine-protein kinase 35
Q9BYX2-2;Q9BYX2;Q9BYX2-3;Q9BYX2-4;Q9BYX2-5;Q9BYX2-6	Isoform 2 of TBC1 domain family member 2A
P27918	Properdin
O75747	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit gamma
O95445	Apolipoprotein M
Q96PY6-3;Q96PY6;Q96PY6-2;Q96PY6-4;Q96PY6-6	Isoform 3 of Serine/threonine-protein kinase Nek1
Q8N2A8	Mitochondrial cardiolipin hydrolase
Q9Y247	Protein FAM50B
Q8TCU4;Q8TCU4-2;Q8TCU4-3	Alstrom syndrome protein 1
Q13596-2	Isoform 1A of Sorting nexin-1
Q8N7Z5	Putative ankyrin repeat domain-containing protein 31
A6NC98;A6NC98-4;A6NC98-6	Coiled-coil domain-containing protein 88B

Q8NFG4-3	Isoform 3 of Folliculin
P27169	Serum paraoxonase/arylesterase 1
P15169	Carboxypeptidase N catalytic chain
Q86VV8;Q86VG3-2;Q86VV8-3;Q9BYX4	Rotatin
P02545;P02545-2	Prelamin-A/C
Q70SY1	Cyclic AMP-responsive element-binding protein 3-like protein 2
P01621	Ig kappa chain V-III region NG9 (Fragment)
Q13257	Mitotic spindle assembly checkpoint protein MAD2A
Q13206	Probable ATP-dependent RNA helicase DDX10
Q08830	Fibrinogen-like protein 1
Q9BRU2	Transcription elongation factor A protein-like 7
Q8WXH0;O60810;P03989;P10075;Q13619;Q13619-2;Q15569;Q5VWM5;Q8WXH0-2;Q8WXH0-7;Q96EF0-2;Q9C029-3;Q9C029-4;Q9NW15;Q9NW15-5;Q9P242-3;Q9ULJ3-2;Q9UMX9;Q9UMX9-2;Q9UMX9-4	Nesprin-2
Q9UMN6	Histone-lysine N-methyltransferase 2B
Q86TL0	Cysteine protease ATG4D
Q5T011-5;Q5T011;Q5T011-4	Isoform 3 of Protein SZT2
Q9UJW7	Zinc finger protein 229
Q8N3X6;Q8N3X6-2	Ligand-dependent nuclear receptor corepressor-like protein
P06753-4;P06753-2;P06753-3;P06753-5	Isoform 4 of Tropomyosin alpha-3 chain
P0CG05;P0CG06	Ig lambda-2 chain C regions
Q8IXJ9	Putative Polycomb group protein ASXL1
P01777	Ig heavy chain V-III region TEI
O43719	HIV Tat-specific factor 1
P49747	Cartilage oligomeric matrix protein
P60174;P60174-1;P60174-4	Triosephosphate isomerase
P04003	C4b-binding protein alpha chain
P55212	Caspase-6
Q9Y2I7	1-phosphatidylinositol 3-phosphate 5-kinase
P62158	Calmodulin
O14628;O14628-4;O14628-5;O14628-6;O14628-7	Zinc finger protein 195
Q8N1I0;Q8N1I0-2;Q8N1I0-3	Dedicator of cytokinesis protein 4
Q96A32	Myosin regulatory light chain 2, skeletal muscle isoform
Q15582	Transforming growth factor-beta-induced protein ig-h3
Q8N6N2	Tetratricopeptide repeat protein 9B
P30485	HLA class I histocompatibility antigen, B-47 alpha chain
O14939-2;O14939;O14939-3	Isoform PLD2B of Phospholipase D2
Q96PZ7;Q96PZ7-2	CUB and sushi domain-containing protein 1
Q96JN8;Q96JN8-2	Neuralized-like protein 4
Q8WW22-2;Q8WW22;Q8WW22-3	Isoform 2 of DnaJ homolog subfamily A member 4
Q9NP86	Calcium-binding protein 5
P19237	Troponin I, slow skeletal muscle
Q86XX4;Q86XX4-2	Extracellular matrix protein FRAS1
P02775	Platelet basic protein
Q96I34	Protein phosphatase 1 regulatory subunit 16A
Q9NS61-2;Q9NS61;Q9NS61-3;Q9NS61-5;Q9NS61-	Isoform 2 of Kv channel-interacting protein 2

6;Q9NS61-7	
P17936;P17936-2	Insulin-like growth factor-binding protein 3
Q9P2M7	Cingulin
P02749	Beta-2-glycoprotein 1
P08603;P08603-2	Complement factor H
Q9ULJ3	Zinc finger and BTB domain-containing protein 21
Q5SRR4-3;Q5SRR4;Q5SRR4-2	Isoform 3 of Lymphocyte antigen 6 complex locus protein G5c
Q15904	V-type proton ATPase subunit S1
A6NK59-3;A6NK59;A6NK59-2	Isoform 3 of Ankyrin repeat and SOCS box protein 14
Q8N3A8-2;Q8N3A8	Isoform 2 of Poly [ADP-ribose] polymerase 8
Q96L91;Q96L91-2;Q96L91-3;Q96L91-4;Q96L91-5	E1A-binding protein p400
P25311	Zinc-alpha-2-glycoprotein
Q5JZY3;Q5JZY3-3	Ephrin type-A receptor 10
Q86VP6-2;Q86VP6	Isoform 2 of Cullin-associated NEDD8-dissociated protein 1
P01613	Ig kappa chain V-I region Ni
P22307-4;P22307;P22307-7;P22307-8	Isoform 4 of Non-specific lipid-transfer protein
Q13620;Q13620-1;Q13620-3	Cullin-4B
Q86TV6;Q86TV6-2	Tetratricopeptide repeat protein 7B
Q5VT98	PRAME family member 20/21
O00592-2;O00592	Isoform 2 of Podocalyxin
Q99542	Matrix metalloproteinase-19
P80748	Ig lambda chain V-III region LOI
P0CF74	Ig lambda-6 chain C region
O60384	Putative zinc finger protein 861
Q9Y5X0;Q9Y5X0-2	Sorting nexin-10
O76080	AN1-type zinc finger protein 5
Q86VF7;Q68D10;Q68D10-2;Q86VF7-2;Q86VF7-3;Q86VF7-4;Q96RN1-3;Q9P1W3;Q9UJM3;Q9UK59	Nebulin-related-anchoring protein
P01591	Immunoglobulin J chain
P01602	Ig kappa chain V-I region HK102 (Fragment)
Q12996	Cleavage stimulation factor subunit 3
Q9NQ89	Uncharacterized protein C12orf4
P01593	Ig kappa chain V-I region AG
Q8IVU3;Q8IVU3-2	Probable E3 ubiquitin-protein ligase HERC6
P09914	Interferon-induced protein with tetratricopeptide repeats 1
Q5HYK7-2;Q5HYK7;Q5HYK7-3;Q5HYK7-4;Q5HYK7-5	Isoform 2 of SH3 domain-containing protein 19
Q9P281	BAH and coiled-coil domain-containing protein 1
Q96J92-3;Q96J92	Isoform 3 of Serine/threonine-protein kinase WNK4
P05155	Plasma protease C1 inhibitor
Q9Y3M2	Protein chibby homolog 1
Q96H12	Myb/SANT-like DNA-binding domain-containing protein 3
Q07954	Prolow-density lipoprotein receptor-related protein 1
Q08380	Galectin-3-binding protein
Q7Z5L0-4	Isoform 4 of Vitelline membrane outer layer protein 1 homolog
Q8IVV2;Q8IVV2-3;Q8IVV2-4;Q8IVV2-5	Lipoxygenase homology domain-containing protein 1
Q562R1	Beta-actin-like protein 2
Q8N9A8	Nuclear envelope phosphatase-regulatory subunit 1

Q7Z5M8;Q7Z5M8-2	Abhydrolase domain-containing protein 12B
Q15027	Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 1
Q8NCT3-3;Q8NCT3;Q8NCT3-2;Q8NCT3-5;Q8NCT3-6	Isoform 3 of Uncharacterized protein KIAA0895
Q96Q15	Serine/threonine-protein kinase SMG1
Q9BZA4	Putative gamma-taxilin-like protein CYorf15B
P37837	Transaldolase
Q86UK0	ATP-binding cassette sub-family A member 12
P01604	Ig kappa chain V-I region Kue
P26927	Hepatocyte growth factor-like protein
Q7Z460;Q7Z460-2;Q7Z460-3	CLIP-associating protein 1
Q15059	Bromodomain-containing protein 3
P12532-2;P12532	Isoform 2 of Creatine kinase U-type, mitochondrial
P02652	Apolipoprotein A-II
P00739-2	Isoform 2 of Haptoglobin-related protein
Q8N8J7	Uncharacterized protein C4orf32
Q8NB78-2;Q8NB78;Q8NB78-4	Isoform 2 of Lysine-specific histone demethylase 1B
P17540	Creatine kinase S-type, mitochondrial
Q9HBY0	NADPH oxidase 3
Q86UQ4	ATP-binding cassette sub-family A member 13
Q96NC0	Zinc finger matrin-type protein 2
Q70EL1;Q70EL1-4;Q70EL1-6;Q70EL1-7	Inactive ubiquitin carboxyl-terminal hydrolase 54
O94911;O94911-3	ATP-binding cassette sub-family A member 8
P35542	Serum amyloid A-4 protein
Q96GS6;Q96GS6-2	Alpha/beta hydrolase domain-containing protein 17A
P0DJI9;P0DJI9-2	Serum amyloid A-2 protein
Q9UPV9	Trafficking kinesin-binding protein 1
Q15485;Q15485-2	Ficolin-2
P04430	Ig kappa chain V-I region BAN
P04070	Vitamin K-dependent protein C
P49643	DNA primase large subunit
P05452	Tetranectin
Q8IX01-4;Q8IX01;Q8IX01-3	Isoform 4 of SURP and G-patch domain-containing protein 2
P55084	Trifunctional enzyme subunit beta, mitochondrial
O14832;O14832-2	Phytanoyl-CoA dioxygenase, peroxisomal
Q8N6F7-3;Q8N6F7;Q8N6F7-2	Isoform 3 of Germinal center-associated signaling and motility protein
A6QL64;A6QL64-4;A6QL64-5;Q8N2N9;Q8N2N9-2;Q8N2N9-3;Q8N2N9-4	Ankyrin repeat domain-containing protein 36A
Q6UX40-2	Isoform 2 of Transmembrane protein 107
Q07837	Neutral and basic amino acid transport protein rBAT
P05156	Complement factor I
Q03001;Q03001-8	Dystonin
Q15782;Q15782-5;Q15782-6	Chitinase-3-like protein 2
P15822	Zinc finger protein 40
Q9BUN8-2;Q9BUN8	Isoform 2 of Derlin-1
Q9H4B7	Tubulin beta-1 chain
P33764	Protein S100-A3
Q7Z7A4-2;Q7Z7A4;Q7Z7A4-4;Q7Z7A4-5;Q7Z7A4-	Isoform 2 of PX domain-containing protein kinase-like protein

6;Q7Z7A4-7	
Q5SZD4	Glycine N-acyltransferase-like protein 3
P21333;P21333-2	Filamin-A
Q9Y3P9;Q9Y3P9-3;Q9Y3P9-4	Rab GTPase-activating protein 1
Q3LI54	Keratin-associated protein 19-8
Q9NU22	Midasin
O43866	CD5 antigen-like
O14647;O14647-2;P62995;P62995-3	Chromodomain-helicase-DNA-binding protein 2
Q96KK5;P04908;P0C0S8;P20671;Q16777;Q6FI13;Q7L7L0; Q93077;Q99878;Q9BTM1	Histone H2A type 1-H
P43121	Cell surface glycoprotein MUC18
Q8TAF3-4;Q8TAF3;Q8TAF3-3	Isoform 4 of WD repeat-containing protein 48
Q69YH5;Q69YH5-2	Cell division cycle-associated protein 2
Q9BZ01	Protein FRG1B
Q12829	Ras-related protein Rab-40B
Q9C091;Q9C091-3	GREB1-like protein
Q96JY0;Q96JY0-2	Protein maelstrom homolog
Q15048	Leucine-rich repeat-containing protein 14
Q13106	Zinc finger protein 154
Q14246-3	Isoform 3 of EGF-like module-containing mucin-like hormone receptor-like 1
Q02985;Q02985-2	Complement factor H-related protein 3
Q96QF0-2;Q96QF0;Q96QF0-8	Isoform 1 of Rab-3A-interacting protein
Q8N3L3	Beta-taxilin
P07910-4;P07910;P07910-2;P07910-3	Isoform 4 of Heterogeneous nuclear ribonucleoproteins C1/C2
Q9Y2M0	Fanconi-associated nuclease 1
Q96S38-2;Q96S38	Isoform 2 of Ribosomal protein S6 kinase delta-1
P80108;P80108-2	Phosphatidylinositol-glycan-specific phospholipase D
Q5TEA6-2	Isoform 2 of Protein sel-1 homolog 2
Q9Y592;Q9Y592-2	Centrosomal protein of 83 kDa
Q8TBP0	TBC1 domain family member 16
O14717-4	Isoform D of tRNA (cytosine(38)-C(5))-methyltransferase
Q5GLZ8;Q5GLZ8-2;Q5GLZ8-3;Q5GLZ8-4	Probable E3 ubiquitin-protein ligase HERC4
Q5VT06	Centrosome-associated protein 350
A6NCD4	Uncharacterized protein C10orf131
P04220	Ig mu heavy chain disease protein
A6NMB1	Sialic acid-binding Ig-like lectin 16
Q8TE96;Q8TE96-3	ATP-dependent RNA helicase DQX1
Q9BRR6;Q9BRR6-2	ADP-dependent glucokinase
Q9HCM2	Plexin-A4
Q8WVY7	Ubiquitin-like domain-containing CTD phosphatase 1
P01612	Ig kappa chain V-I region Mev
Q86TA1	MOB kinase activator 3B
P22105;P22105-2;Q16473	Tenascin-X
Q01433-3;Q01433;Q01433-2;Q01433-4;Q01433-5	Isoform Ex1A-3 of AMP deaminase 2
O95819-5;O95819-4	Isoform 5 of Mitogen-activated protein kinase kinase kinase kinase 4
O95819-2;O95819;O95819-3	Isoform 2 of Mitogen-activated protein kinase kinase kinase kinase 4
Q6ZMR5-2	Isoform 2 of Transmembrane protease serine 11A

P46939-2;P46939	Isoform 2 of Utrophin
Q5JRA6;Q5JRA6-2	Melanoma inhibitory activity protein 3
Q5VWZ2	Lysophospholipase-like protein 1
Q9BT09	Protein canopy homolog 3
A4UGR9;A4UGR9-2;A4UGR9-3;O75167;O75167-2;O75167-4;O75167-5	Xin actin-binding repeat-containing protein 2
Q7L523;Q5VZM2;Q5VZM2-2	Ras-related GTP-binding protein A
P07711	Cathepsin L1
Q99576-3	Isoform 2 of TSC22 domain family protein 3
Q9HB21	Pleckstrin homology domain-containing family A member 1
P01774;P01770	Ig heavy chain V-III region POM
O15119;O15119-2;O15119-3	T-box transcription factor TBX3
Q9UL03-3;P51814-6;P51814-8;Q13398;Q13398-3;Q13398-4;Q13398-5;Q13398-6;Q13398-7;Q5JVG2;Q5JVG2-2;Q5JVG2-3;Q5TBE3;Q5TBE3-2;Q9UL03	Isoform 3 of Integrator complex subunit 6
Q9NXU5	ADP-ribosylation factor-like protein 15
Q9Y6B6	GTP-binding protein SAR1b
Q9NYP9	Protein Mis18-alpha
Q86VF2-5;Q86VF2	Isoform 5 of Immunoglobulin-like and fibronectin type III domain-containing protein 1
P60660-2;P60660	Isoform Smooth muscle of Myosin light polypeptide 6
P01776	Ig heavy chain V-III region WAS
O15068-3;O15068-2;O15068-5	Isoform 3 of Guanine nucleotide exchange factor DBS
Q9Y608;Q9Y608-2;Q9Y608-4	Leucine-rich repeat flightless-interacting protein 2
Q4LDR2	Cortexin-3
P17948;P17948-5;P17948-6;P17948-7;P17948-8	Vascular endothelial growth factor receptor 1
Q9NX57	Ras-related protein Rab-20
Q9UJW3;Q9UJW3-2	DNA (cytosine-5)-methyltransferase 3-like
A8MXY4	Zinc finger protein 99
Q9Y6V0;Q9Y6V0-2;Q9Y6V0-3	Protein piccolo
P02654	Apolipoprotein C-I
Q9NZL9;Q9NZL9-2;Q9NZL9-3;Q9NZL9-4;Q9NZL9-5	Methionine adenosyltransferase 2 subunit beta
P60709	Actin, cytoplasmic 1
Q66GS9;Q66GS9-2	Centrosomal protein of 135 kDa
Q16610-3	Isoform 3 of Extracellular matrix protein 1
Q86VM9;Q86VM9-2	Zinc finger CCCH domain-containing protein 18
Q96P20-4;Q96P20;Q96P20-2;Q96P20-3;Q96P20-5	Isoform 4 of NACHT, LRR and PYD domains-containing protein 3
Q8N103	T-cell activation Rho GTPase-activating protein
Q5T0J7-2	Isoform 2 of Testis-expressed sequence 35 protein
O15056;O15056-2;O15056-3	Synaptojanin-2
P04211	Ig lambda chain V region 4A
Q9NYT6-2	Isoform 2 of Zinc finger protein 226
Q6UWQ7;Q6UWQ7-2	Insulin growth factor-like family member 2
P55058	Phospholipid transfer protein
P02787	Serotransferrin
Q53SF7;Q53SF7-2;Q53SF7-3;Q53SF7-4;Q53SF7-5	Cordon-bleu protein-like 1
Q14257	Reticulocalbin-2

O15540	Fatty acid-binding protein, brain
Q9Y4W2;Q9Y4W2-2;Q9Y4W2-3;Q9Y4W2-4	Ribosomal biogenesis protein LAS1L
Q15166	Serum paraoxonase/lactonase 3
Q3SY69-3;Q3SY69	Isoform 3 of Mitochondrial 10-formyltetrahydrofolate dehydrogenase
Q8TC44-2;Q8TC44	Isoform 2 of POC1 centriolar protein homolog B
Q6IN84-2;Q6IN84	Isoform 2 of rRNA methyltransferase 1, mitochondrial
Q9Y3D2	Methionine-R-sulfoxide reductase B2, mitochondrial
Q96RD9;Q96RD9-2;Q96RD9-3;Q96RD9-4;Q96RD9-5	Fc receptor-like protein 5
P51884	Lumican
P01614	Ig kappa chain V-II region Cum
Q3KQU3;Q3KQU3-2;Q3KQU3-3;Q3KQU3-4	MAP7 domain-containing protein 1
P01876	Ig alpha-1 chain C region
Q9HCG1	Zinc finger protein 160
Q6ZS86	Putative glycerol kinase 5
Q96A08;O60814;P06899;P23527;P33778;P57053;P58876;P62807;Q16778;Q5QNW6;Q5QNW6-2;Q8N257;Q93079;Q99877;Q99879;Q99880	Histone H2B type 1-A
Q8NAT9	Putative uncharacterized protein encoded by LINC00324
Q8NCU1;Q8NCU1-2	Uncharacterized protein encoded LINC00521
P01625	Ig kappa chain V-IV region Len
Q96FC7-3	Isoform 3 of Phytanoyl-CoA hydroxylase-interacting protein-like
Q9UNH5;Q9UNH5-2;Q9UNH5-3;Q9UNH5-4;Q9UNH5-5	Dual specificity protein phosphatase CDC14A
Q96IY4;Q96IY4-2	Carboxypeptidase B2
Q9HC96;Q9HC96-2;Q9HC96-3;Q9HC96-4;Q9HC96-5	Calpain-10
P02545-4	Isoform 4 of Prelamin-A/C
P02741;P02741-2	C-reactive protein
P01019	Angiotensinogen
Q13884;Q13884-2	Beta-1-syntrophin
Q86XW9	Thioredoxin domain-containing protein 6
Q9UK55	Protein Z-dependent protease inhibitor
P62736;P63267;P63267-2	Actin, aortic smooth muscle
Q86U86;Q86U86-2;Q86U86-3;Q86U86-4;Q86U86-5;Q86U86-6;Q86U86-7;Q86U86-8;Q86U86-9	Protein polybromo-1
Q02750-2;Q02750	Isoform 2 of Dual specificity mitogen-activated protein kinase kinase 1
Q13790	Apolipoprotein F
P22105-3	Isoform 3 of Tenascin-X
Q6P2N6;P33765-2	Protein ADORA3, isoform 3
P42263;P42263-2	Glutamate receptor 3
P02751-5	Isoform 5 of Fibronectin
Q6ZN16;Q6ZN16-2;Q6ZN16-3	Mitogen-activated protein kinase kinase kinase 15
Q9H227;Q9H227-2	Cytosolic beta-glucosidase
Q96EF0	Myotubularin-related protein 8
Q93062-4;Q93062;Q93062-2;Q93062-3;Q93062-5	Isoform D of RNA-binding protein with multiple splicing
Q14624-2	Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H4
Q14624;Q14624-3;Q14624-4	Inter-alpha-trypsin inhibitor heavy chain H4
P01834	Ig kappa chain C region
P01009;P01009-2;P01009-3;Q9Y238;Q9Y238-3	Alpha-1-antitrypsin

Q9Y2L1;Q9Y2L1-2	Exosome complex exonuclease RRP44
Q5JPF3;Q5JPF3-2	Ankyrin repeat domain-containing protein 36C
Q04756	Hepatocyte growth factor activator
Q96BN8	Ubiquitin thioesterase otulin
Q9P2K5;Q9P2K5-2;Q9P2K5-3	Myelin expression factor 2
P35240-4	Isoform 4 of Merlin
Q68DL7	Uncharacterized protein C18orf63
P04196	Histidine-rich glycoprotein
Q9H251-8;Q9H251;Q9H251-2;Q9H251-3;Q9H251-4;Q9H251-7;Q9H251-9	Isoform 8 of Cadherin-23
Q9UHQ1;Q9UHQ1-3;Q9UHQ1-4	Nuclear prelamin A recognition factor
Q4W5P6	Protein TMEM155
Q9Y5J7	Mitochondrial import inner membrane translocase subunit Tim9
Q68DA7;Q68DA7-2	Formin-1
Q68G75;Q68G75-4;Q68G75-5	LEM domain-containing protein 1
Q9BYG4-2;Q9BYG4	Isoform 2 of Partitioning defective 6 homolog gamma
Q8TDC3;Q8TDC3-2	Serine/threonine-protein kinase BRSK1
Q9NY65;Q9NY65-2	Tubulin alpha-8 chain
Q6W2J9;Q6W2J9-2;Q6W2J9-3;Q6W2J9-4	BCL-6 corepressor
Q9NQW1-5;Q9NQW1;Q9NQW1-2;Q9NQW1-4	Isoform 5 of Protein transport protein Sec31B
P00751-2	Isoform 2 of Complement factor B
Q8N4L1	Transmembrane protein 151A
Q8NC06-2	Isoform 2 of Acyl-CoA-binding domain-containing protein 4
Q96T76;Q96B36;Q96B36-2;Q96B36-3;Q96T76-5;Q96T76-8;Q96T76-9	MMS19 nucleotide excision repair protein homolog
P32456	Interferon-induced guanylate-binding protein 2
Q92583	C-C motif chemokine 17
Q8TCZ2;Q8TCZ2-2;Q8TCZ2-3;Q8TCZ2-5;Q8TCZ2-6	CD99 antigen-like protein 2
Q14520;Q14520-2	Hyaluronan-binding protein 2
Q2M3A8	Putative uncharacterized protein MRGPRG-AS1
P01814	Ig heavy chain V-II region OU
P04438	Ig heavy chain V-II region SESS
P53004	Biliverdin reductase A
P01763	Ig heavy chain V-III region WEA
Q8IYW4	ENTH domain-containing protein 1
Q7L1T6	Cytochrome b5 reductase 4
Q16836;Q16836-2;Q16836-3	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial
Q3LI73	Keratin-associated protein 19-4
P01024;A5PL33;A5PL33-2;O75110;O75110-2;Q92833-2;Q969U6;Q969U6-2;Q9NTW7-2;Q9NZV5;Q9NZV5-2;Q9P202;Q9P202-2	Complement C3
P02763	Alpha-1-acid glycoprotein 1
Q96G28;Q96G28-2	Coiled-coil domain-containing protein 104
Q9NYZ1;Q96ET8;Q96ET8-2;Q96ET8-3	Golgi apparatus membrane protein TVP23 homolog B
P63261	Actin, cytoplasmic 2
Q8NA77	Testis-expressed sequence 19 protein
B9A064;P0CG04	Immunoglobulin lambda-like polypeptide 5

P07355;P07355-2	Annexin A2
Q7Z4G4;Q7Z4G4-2;Q7Z4G4-3	tRNA (guanine(10)-N2)-methyltransferase homolog
Q8IUX4-2	Isoform 2 of DNA dC->dU-editing enzyme APOBEC-3F
P01781;P01768;P01772;P01773	Ig heavy chain V-III region GAL
P01775	Ig heavy chain V-III region LAY
P02747	Complement C1q subcomponent subunit C
Q99795	Cell surface A33 antigen
O00187	Mannan-binding lectin serine protease 2
P01620	Ig kappa chain V-III region SIE
Q3ZCT8;Q3ZCT8-2	Kelch repeat and BTB domain-containing protein 12
P01716	Ig lambda chain V-IV region X
Q92833;Q92833-3	Protein Jumonji
O95236;O95236-2	Apolipoprotein L3
P02730	Band 3 anion transport protein
P30626	Sorcin
Q9Y490	Talin-1
P02675	Fibrinogen beta chain
Q5TH69	Brefeldin A-inhibited guanine nucleotide-exchange protein 3
Q9NR99	Matrix-remodeling-associated protein 5
Q9Y3E7-3	Isoform 3 of Charged multivesicular body protein 3
Q9C019	Tripartite motif-containing protein 15
Q9Y6X9	MORC family CW-type zinc finger protein 2
O76041	Nebulette
Q9UPN3;Q9NZC7;Q9NZC7-2;Q9UPN3-2;Q9UPN3-3;Q9UPN3-4;Q9UPN3-5	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
O60636-2;O60636	Isoform 2 of Tetraspanin-2
B6A8C7;B6A8C7-2	T-cell-interacting, activating receptor on myeloid cells protein 1
P01699;P01709	Ig lambda chain V-I region VOR
P52848;P52848-2	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 1
P02647;Q5JR98;Q6V1P9-3;Q6V1P9-4;Q8N2G4-2;Q9HB71;Q9Y5F6;Q9Y5F6-2	Apolipoprotein A-I
Q9BSK4	Protein fem-1 homolog A
Q11203-13	Isoform B8 of CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase
Q86X27	Ras-specific guanine nucleotide-releasing factor RalGPS2
P80723;P80723-2	Brain acid soluble protein 1
P61296	Heart- and neural crest derivatives-expressed protein 2
Q9Y2K3	Myosin-15
Q9BXR6	Complement factor H-related protein 5
P05546	Heparin cofactor 2
Q9Y2D5-6;Q9Y2D5;Q9Y2D5-4;Q9Y2D5-5;Q9Y2D5-7	Isoform 4 of A-kinase anchor protein 2
Q9HAE3;Q9HAE3-2	EF-hand calcium-binding domain-containing protein 1
Q9GZM8-3;Q9GZM8;Q9GZM8-2	Isoform 3 of Nuclear distribution protein nudE-like 1
Q96CJ1;Q96JC9	ELL-associated factor 2
Q5T6S3-2	Isoform 2 of PHD finger protein 19
O95292-2	Isoform 2 of Vesicle-associated membrane protein-associated protein B/C
Q562F6;Q562F6-2;Q562F6-3	Shugoshin-like 2

Q6ZU64	Coiled-coil domain-containing protein 108
P35240;P35240-10;P35240-2;P35240-3;P35240-5;P35240-6;P35240-7;P35240-8;P35240-9	Merlin
Q8NEY1-4;Q8NEY1;Q8NEY1-2;Q8NEY1-3;Q8NEY1-7	Isoform 4 of Neuron navigator 1
Q9NUM4	Transmembrane protein 106B
Q5T4S7-2;Q5T4S7;Q5T4S7-3;Q5T4S7-4	Isoform 2 of E3 ubiquitin-protein ligase UBR4
Q17R31-5;Q17R31;Q17R31-2;Q17R31-3	Isoform 5 of Putative deoxyribonuclease TATDN3
Q16625-3;Q16625;Q16625-2;Q16625-4;Q16625-5	Isoform 3 of Occludin
P01605	Ig kappa chain V-I region Lay
P14550	Alcohol dehydrogenase [NADP(+)]
Q8WWL2-4;Q8WWL2;Q8WWL2-3	Isoform 4 of Protein spire homolog 2
Q9NRU3;Q9NRU3-2	Metal transporter CNNM1
Q9BQF6;Q9BQF6-2;Q9BQF6-4;Q9BQF6-5	Sentrin-specific protease 7
Q5RHP9	Uncharacterized protein C1orf173
Q9NVQ4-3;Q9NVQ4;Q9NVQ4-2	Isoform 3 of Fas apoptotic inhibitory molecule 1
P07339	Cathepsin D
Q9H2R5;Q9H2R5-2;Q9H2R5-3;Q9H2R5-4	Kallikrein-15
Q5T1B0;Q5T1B0-3	Axonemal dynein light chain domain-containing protein 1
P20700	Lamin-B1
Q8TBX8;Q8TBX8-2;Q8TBX8-3	Phosphatidylinositol 5-phosphate 4-kinase type-2 gamma
P02679;P02679-2	Fibrinogen gamma chain
P01780	Ig heavy chain V-III region JON
Q8ND04-2;Q8ND04;Q8ND04-3	Isoform 2 of Protein SMG8
Q14517;Q9HCL0;Q9HCL0-2	Protocadherin Fat 1
Q9BX66-12	Isoform 12 of Sorbin and SH3 domain-containing protein 1
Q8WW59	SPRY domain-containing protein 4
Q8IWK6;Q8IWK6-3	Probable G-protein coupled receptor 125
P08697;P08697-2	Alpha-2-antiplasmin
P01608	Ig kappa chain V-I region Roy
Q8NF91-2	Isoform 2 of Nesprin-1
P04433	Ig kappa chain V-III region VG (Fragment)
P82930	28S ribosomal protein S34, mitochondrial
P23458	Tyrosine-protein kinase JAK1
P02655	Apolipoprotein C-II
A6NEE1	Pleckstrin homology domain-containing family D member 1
Q9ULD2;Q9ULD2-2	Microtubule-associated tumor suppressor 1
Q8TF76;Q8TF76-2	Serine/threonine-protein kinase haspin
Q8N145	Leucine-rich repeat LGI family member 3
Q6ZSB3	Putative uncharacterized protein encoded by LINC00299
P07099	Epoxide hydrolase 1
Q8WXF8-2;Q8WXF8	Isoform 2 of DNA-binding death effector domain-containing protein 2
Q15391	P2Y purinoceptor 14
Q4V328-2	Isoform 2 of GRIP1-associated protein 1
P01766	Ig heavy chain V-III region BRO
P62805	Histone H4
P01034	Cystatin-C
P02751-2	Isoform 2 of Fibronectin

O00161	Synaptosomal-associated protein 23
Q9NQ79-3;Q9NQ79;Q9NQ79-2	Isoform 3 of Cartilage acidic protein 1
P10643	Complement component C7
Q9HD20-2;Q9HD20	Isoform B of Probable cation-transporting ATPase 13A1
P47710;P47710-2;P47710-3;P47710-4	Alpha-S1-casein
P61587	Rho-related GTP-binding protein RhoE
Q9Y283;Q9Y283-2	Inversin
O95486	Protein transport protein Sec24A
Q15195	Plasminogen-like protein A
Q9H8M2;Q9H8M2-1;Q9H8M2-2;Q9H8M2-3;Q9H8M2-6	Bromodomain-containing protein 9
P01877	Ig alpha-2 chain C region
Q96PH6	Beta-defensin 118
Q9HD23-3	Isoform 3 of Magnesium transporter MRS2 homolog, mitochondrial
Q9H5I5;Q9H5I5-2;Q9H5I5-3;Q9H5I5-4	Piezo-type mechanosensitive ion channel component 2
P06396-3	Isoform 3 of Gelsolin
P06396;P06396-2;Q9P1A2	Gelsolin
P02768;A6NJZ7;A6NNM3;O00339;O00339-2;O00339-3;O00339-4;P02768-2;P17927;Q92985-3;Q9H1A7;Q9UFD9;Q9UFV3;Q9UJ41;Q9UJ41-2;Q9UJ41-3;Q9Y3S1	Serum albumin
P20929	Nebulin
P00738	Haptoglobin
P61626	Lysozyme C
P00558	Phosphoglycerate kinase 1
Q9P2S6;Q9P2S6-2;Q9P2S6-3;Q9P2S6-4	Ankyrin repeat and MYND domain-containing protein 1
P02745	Complement C1q subcomponent subunit A
P02649	Apolipoprotein E
Q9BZD3;P0CAP2;P0CAP2-3	Putative GRINL1B complex locus protein 2
Q5T0Z8;Q5T0Z8-2;Q5T0Z8-4	Uncharacterized protein C6orf132
Q6UVJ0	Spindle assembly abnormal protein 6 homolog
Q4V328;Q4V328-3;Q4V328-4	GRIP1-associated protein 1
P07225	Vitamin K-dependent protein S
P03986	T-cell receptor gamma-2 chain C region
P22692	Insulin-like growth factor-binding protein 4
P48740-2;P48740-4	Isoform 2 of Mannan-binding lectin serine protease 1
P01594	Ig kappa chain V-I region AU
P32119	Peroxiredoxin-2
Q8N9V7	Testis- and ovary-specific PAZ domain-containing protein 1
Q8N1V2;Q8N1V2-2;Q8N1V2-3	WD repeat-containing protein 16
P08253	72 kDa type IV collagenase
Q9P2P6;Q9P2P6-3	StAR-related lipid transfer protein 9
P07358	Complement component C8 beta chain
P01011-3	Isoform 3 of Alpha-1-antichymotrypsin
Q9UGM5;Q9UGM5-2	Fetuin-B
P55916-2;P55916;P55916-3	Isoform UCP3S of Mitochondrial uncoupling protein 3
P20042	Eukaryotic translation initiation factor 2 subunit 2
Q9BRP4;Q9BRP4-2;Q9BRP4-3	Proteasomal ATPase-associated factor 1

Q8N4S0	Coiled-coil domain-containing protein 82
O15151;O15151-2;O15151-3;O15151-5	Protein Mdm4
P03952	Plasma kallikrein
Q9UJX3;Q9UJX3-2	Anaphase-promoting complex subunit 7
P50539;P50539-2;P50539-3;P50539-4	Max-interacting protein 1
Q13137-3;Q13137;Q13137-2;Q13137-4	Isoform 3 of Calcium-binding and coiled-coil domain-containing protein 2
P01597	Ig kappa chain V-I region DEE
Q9NZH6	Interleukin-37
P06331	Ig heavy chain V-II region ARH-77
Q9Y4D8;Q9Y4D8-4	Probable E3 ubiquitin-protein ligase HECTD4
Q6Q4G3;Q6Q4G3-2	Aminopeptidase Q
Q6ZS81-4;Q6ZS81-3;Q6ZS81-5	Isoform 4 of WD repeat- and FYVE domain-containing protein 4
O76003	Glutaredoxin-3
Q9UNE2-2;Q9UNE2	Isoform 2 of Rab effector Noc2
Q16655	Melanoma antigen recognized by T-cells 1
O00411	DNA-directed RNA polymerase, mitochondrial
P24592	Insulin-like growth factor-binding protein 6
Q86Y33-5;Q86Y33-4	Isoform 5 of Cell division cycle protein 20 homolog B
Q99538	Legumain
P01596	Ig kappa chain V-I region CAR
Q4J6C6;Q4J6C6-2;Q4J6C6-3;Q4J6C6-4	Prolyl endopeptidase-like
Q6ZWH5;Q6ZWH5-2;Q6ZWH5-3;Q6ZWH5-4	Serine/threonine-protein kinase Nek10
Q7L273	BTB/POZ domain-containing protein KCTD9
Q6ZMU5;Q6ZMU5-2	Tripartite motif-containing protein 72
O15265-3;O15265	Isoform 3 of Ataxin-7
Q9NRG7-3	Isoform 3 of Epimerase family protein SDR39U1
Q6Y7W6;Q6Y7W6-3;Q6Y7W6-4	PERQ amino acid-rich with GYF domain-containing protein 2
Q6ZN54-5;Q6ZN54;Q6ZN54-3;Q6ZN54-4;Q6ZN54-6	Isoform 5 of Differentially expressed in FDCP 8 homolog
Q8WXX0	Dynein heavy chain 7, axonemal
Q53RE8	Ankyrin repeat domain-containing protein 39
Q5U5Z8;Q5U5Z8-2;Q5U5Z8-3	Cytosolic carboxypeptidase 2
Q9Y4M8	Putative uncharacterized protein encoded by LINC00588
P63123	HERV-K_1q23.3 provirus ancestral Pro protein
Q96QB1;Q96QB1-3;Q96QB1-5	Rho GTPase-activating protein 7
Q96HD9	Aspartoacylase-2
O75362	Zinc finger protein 217
P67936	Tropomyosin alpha-4 chain
P01833	Polymeric immunoglobulin receptor
Q96MG8;Q96MG8-2	Protein-L-isoaspartate O-methyltransferase domain-containing protein 1
A1A4V9;A1A4V9-2;A1A4V9-4	Coiled-coil domain-containing protein C16orf93
P01700	Ig lambda chain V-I region HA
P02765	Alpha-2-HS-glycoprotein
P36955	Pigment epithelium-derived factor
Q6P9F0;P80362;Q6P9F0-2	Coiled-coil domain-containing protein 62
Q9H6R6-2;Q9H6R6	Isoform 2 of Palmitoyltransferase ZDHHC6
Q86UD1	Out at first protein homolog
Q14714	Sarcospan

Q92834;Q92834-2;Q92834-4	X-linked retinitis pigmentosa GTPase regulator
P0C0L5	Complement C4-B
P0C0L4;P0C0L4-2	Complement C4-A
P62263	40S ribosomal protein S14
Q99766-3;Q99766;Q99766-2	Isoform 3 of ATP synthase subunit s, mitochondrial
Q6NUN0;Q6NUN0-2	Acyl-coenzyme A synthetase ACSM5, mitochondrial
Q9H269	Vacuolar protein sorting-associated protein 16 homolog
Q9H6H4	Receptor expression-enhancing protein 4
Q9NZJ4	Sacsin
Q8TF21;Q8TF21-2	Ankyrin repeat domain-containing protein 24
Q15084-2;Q15084	Isoform 2 of Protein disulfide-isomerase A6
O75290-2	Isoform 2 of Zinc finger protein 780A
Q8IZD9	Dedicator of cytokinesis protein 3
Q8N6G6;Q8N6G6-1;Q8N6G6-2;Q8N6G6-4	ADAMTS-like protein 1
P68400;Q8NEV1	Casein kinase II subunit alpha
Q8TC20-5;Q8TC20;Q8TC20-2;Q8TC20-3;Q8TC20-4	Isoform 5 of Cancer-associated gene 1 protein
Q6JVE9-2;Q6JVE9	Isoform 2 of Epididymal-specific lipocalin-8
P10720	Platelet factor 4 variant
P02751-12	Isoform 12 of Fibronectin
Q12805-4;Q12805;Q12805-2;Q12805-3	Isoform 4 of EGF-containing fibulin-like extracellular matrix protein 1
Q9P1Q0-3;Q9P1Q0;Q9P1Q0-2;Q9P1Q0-4	Isoform 3 of Vacuolar protein sorting-associated protein 54
Q5SZK8	FRAS1-related extracellular matrix protein 2
Q3KRB8	Rho GTPase-activating protein 11B
O00391;O00391-2	Sulfhydryl oxidase 1
Q76N89	E3 ubiquitin-protein ligase HECW1
Q9H0M0-3;Q9H0M0	Isoform 3 of NEDD4-like E3 ubiquitin-protein ligase WWP1
Q8IUF8-4;Q8IUF8	Isoform 4 of Bifunctional lysine-specific demethylase and histidyl-hydroxylase MINA
P48426	Phosphatidylinositol 5-phosphate 4-kinase type-2 alpha
Q9HAW4;Q9HAW4-2;Q9HAW4-3	Claspin
Q96N21;Q96N21-2	AP-4 complex accessory subunit tepsin
Q8WXW3	Progesterone-induced-blocking factor 1
Q96L33	Rho-related GTP-binding protein RhoV
Q8IWJ2;Q8IWJ2-3	GRIP and coiled-coil domain-containing protein 2
O60308;O60308-3	Centrosomal protein of 104 kDa
Q7Z4T9;Q7Z4T9-3;Q7Z4T9-4;Q7Z4T9-5;Q7Z4T9-6;Q7Z4T9-7;Q7Z4T9-8	Protein MAATS1
Q9UNH7	Sorting nexin-6
Q5TB80;Q5TB80-2	Centrosomal protein of 162 kDa
Q99972	Myocilin
Q14204	Cytoplasmic dynein 1 heavy chain 1
O95155-4;O95155;O95155-2	Isoform 4 of Ubiquitin conjugation factor E4 B
Q8TCI5;Q8TCI5-3	Protein pitchfork
P35680-2;P35680-4	Isoform B of Hepatocyte nuclear factor 1-beta
P19652	Alpha-1-acid glycoprotein 2
P13667	Protein disulfide-isomerase A4
P01031	Complement C5

Q8N2Y8	Iporin
Q969M2	Gap junction alpha-10 protein
P55290;P55290-2;P55290-3;P55290-4	Cadherin-13
Q9H161	Homeobox protein aristaless-like 4
Q8IVG5	Sterile alpha motif domain-containing protein 9-like
Q8WUF8-2;Q8WUF8	Isoform 2 of Protein FAM172A
O43593;O43593-2	Protein hairless
Q9BYX7	Putative beta-actin-like protein 3
Q14BN4;Q14BN4-6	Sarcolemmal membrane-associated protein
Q9NV72	Zinc finger protein 701
O15296-4;O15296;O15296-2	Isoform D of Arachidonate 15-lipoxygenase B
O43463	Histone-lysine N-methyltransferase SUV39H1
P04217	Alpha-1B-glycoprotein
P49643-2	Isoform 2 of DNA primase large subunit
B0I1T2-4;B0I1T2-3	Isoform 4 of Unconventional myosin-Ig
H7BZ55	Putative ciliary rootlet coiled-coil protein-like 3 protein
Q9NUZ1-2;Q9NUZ1;Q9NUZ1-4	Isoform 2 of Acyl-coenzyme A oxidase-like protein
P00748	Coagulation factor XII
Q9BQI0;Q9BQI0-2;Q9BQI0-3	Allograft inflammatory factor 1-like
P08514-3;P08514;P08514-2	Isoform 3 of Integrin alpha-IIb
Q9H5Z6;Q9H5Z6-2	Protein FAM124B
Q5JST6	EF-hand domain-containing family member C2
Q5T7R7	Uncharacterized protein C1orf185
Q96LK0	Centrosomal protein of 19 kDa
P0CG34;P0CG35	Thymosin beta-15A
P53634;P53634-2;P53634-3	Dipeptidyl peptidase 1
P00709	Alpha-lactalbumin
Q9BXL5	Hemogen
Q9BSL1	Ubiquitin-associated domain-containing protein 1
O14929	Histone acetyltransferase type B catalytic subunit
Q86UX7;Q86UX7-2	Fermitin family homolog 3
O15061;O15061-2	Synemin
P01011;P01011-2	Alpha-1-antichymotrypsin
Q86TI4	WD repeat-containing protein 86
P50583	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]
Q15025;Q15025-2;Q15025-3	TNFAIP3-interacting protein 1
Q9UM11-3;Q9UM11;Q9UM11-2	Isoform 3 of Fizzy-related protein homolog
Q5BJE1-2;Q5BJE1;Q5BJE1-3;Q5BJE1-4	Isoform 2 of Coiled-coil domain-containing protein 178
P49771-2	Isoform 2 of Fms-related tyrosine kinase 3 ligand
Q6V1P9	Protocadherin-23
Q9P2E3	NFX1-type zinc finger-containing protein 1
P03950	Angiogenin
Q9BZ81	Melanoma-associated antigen B5
O75298-2;O75298	Isoform RTN2-B of Reticulon-2
P63241-2;P63241	Isoform 2 of Eukaryotic translation initiation factor 5A-1
Q86VQ0	Lebercilin
P68871	Hemoglobin subunit beta

Q16584	Mitogen-activated protein kinase kinase kinase 11
P37802	Transgelin-2
Q6ZMP0	Thrombospondin type-1 domain-containing protein 4
Q16649	Nuclear factor interleukin-3-regulated protein
Q09666	Neuroblast differentiation-associated protein AHNAK
Q9P1Z9;Q9P1Z9-2;Q9P1Z9-3	Coiled-coil domain-containing protein 180
P20851;P20851-2	C4b-binding protein beta chain
Q8N4C6-2;P0C7N5;Q8N4C6;Q8N4C6-10;Q8N4C6-4;Q8N4C6-5;Q8N4C6-7;Q8N4C6-9	Isoform 2 of Ninein
Q8NFU5	Inositol polyphosphate multikinase
P05062	Fructose-bisphosphate aldolase B
P61244-2;P61244	Isoform 2 of Protein max
P00740-2	Isoform 2 of Coagulation factor IX
Q9Y3C1-2	Isoform 2 of Nucleolar protein 16
Q9BXW6;Q9BXW6-4	Oxysterol-binding protein-related protein 1
Q15438-2;Q15438	Isoform 2 of Cytohesin-1
O43236-2;O43236;O43236-3;O43236-4	Isoform 2 of Septin-4
O15539;O15539-2;O15539-3	Regulator of G-protein signaling 5
P01701	Ig lambda chain V-I region NEW
P43897;P43897-2;P43897-3;P43897-4	Elongation factor Ts, mitochondrial
Q71U36	Tubulin alpha-1A chain
P15248	Interleukin-9
P41227-2;P41227	Isoform 2 of N-alpha-acetyltransferase 10
Q96CW5-2;Q96CW5;Q96CW5-3	Isoform 2 of Gamma-tubulin complex component 3
O75688	Protein phosphatase 1B
P01112-2;P01112	Isoform 2 of GTPase HRas
Q149M9;Q149M9-3	NACHT and WD repeat domain-containing protein 1
Q9NWS1;Q9NWS1-3;Q9NWS1-4;Q9NWS1-6;Q9NWS1-7	PCNA-interacting partner
P61769	Beta-2-microglobulin
Q8HWS3	DNA-binding protein RFX6
P08185	Corticosteroid-binding globulin
P25874	Mitochondrial brown fat uncoupling protein 1
P08519	Apolipoprotein(a)
Q86YR7-2;Q86YR7;Q86YR7-4	Isoform 2 of Probable guanine nucleotide exchange factor MCF2L2
P0CG38;P0CG39	POTE ankyrin domain family member I
P04083	Annexin A1
Q13907;Q13907-2	Isopentenyl-diphosphate Delta-isomerase 1
P00488	Coagulation factor XIII A chain
P35030;P35030-2;P35030-3;P35030-4	Trypsin-3
Q8IUX4;Q9HC16-3	DNA dC->dU-editing enzyme APOBEC-3F
Q9P0N8-2;Q9P0N8	Isoform 2 of E3 ubiquitin-protein ligase MARCH2
Q4G0P3;Q4G0P3-10;Q4G0P3-5;Q4G0P3-6;Q4G0P3-8	Hydrocephalus-inducing protein homolog
Q658T7	Putative protein FAM90A2P
Q9UF72	Putative TP73 antisense gene protein 1
P18510-4;P18510;P18510-2;P18510-3	Isoform 4 of Interleukin-1 receptor antagonist protein
Q99645	Epiphycan
O75037;O75037-2;O75037-3;O75037-4	Kinesin-like protein KIF21B

P43652	Afamin
Q9HCE1	Putative helicase MOV-10
P60606	Cortexin-1
P08571	Monocyte differentiation antigen CD14
Q8IWS0;Q8IWS0-3	PHD finger protein 6
Q9UKG9;Q9UKG9-2;Q9UKG9-3	Peroxisomal carnitine O-octanoyltransferase
P58005;P58005-2;P58005-3	Sestrin-3
Q9H4A6	Golgi phosphoprotein 3
P01765	Ig heavy chain V-III region TIL
O15265-2	Isoform b of Ataxin-7
P05160	Coagulation factor XIII B chain
A6H8Y1-6;A6H8Y1;A6H8Y1-2;A6H8Y1-3;A6H8Y1-4;A6H8Y1-5;A6H8Y1-7	Isoform 6 of Transcription factor TFIIIB component B" homolog
P00450	Ceruloplasmin
Q14508;Q14508-2;Q14508-3	WAP four-disulfide core domain protein 2
Q9NTW7	Zinc finger protein 64 homolog, isoforms 3 and 4
Q59EK9;Q59EK9-2;Q59EK9-3;Q59EK9-4	RUN domain-containing protein 3A
Q96M02-2;Q96M02	Isoform 2 of Centrosomal protein C10orf90
Q13115;Q13115-2	Dual specificity protein phosphatase 4
P35612;P35612-2;P35612-3;P35612-4;P35612-5;P35612-6;P35612-7;P35612-8	Beta-adducin
Q8IXZ2	Zinc finger CCCH domain-containing protein 3
Q8TB03	Uncharacterized protein CXorf38
Q96BM9	ADP-ribosylation factor-like protein 8A
Q9UKE5;Q9UKE5-2;Q9UKE5-3;Q9UKE5-4;Q9UKE5-5;Q9UKE5-6;Q9UKE5-7;Q9UKE5-8	TRAF2 and NCK-interacting protein kinase
O15021;O15021-2;O15021-3	Microtubule-associated serine/threonine-protein kinase 4
O15021-1	Isoform 5 of Microtubule-associated serine/threonine-protein kinase 4
P06727;O95236-3	Apolipoprotein A-IV
Q99944	Epidermal growth factor-like protein 8
P38919	Eukaryotic initiation factor 4A-III
O75122	CLIP-associating protein 2
Q8N8F7;Q8N8F7-2	Leucine-rich single-pass membrane protein 1
P01779	Ig heavy chain V-III region TUR
P18206-2;P18206	Isoform 1 of Vinculin
Q8IV77	Cyclic nucleotide-gated cation channel alpha-4
Q14247-2	Isoform 2 of Src substrate cortactin
P52952	Homeobox protein Nkx-2.5
Q15057	Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2
Q6IEG0	U11/U12 small nuclear ribonucleoprotein 48 kDa protein
Q96T66	Nicotinamide mononucleotide adenylyltransferase 3
Q9UBS9-2	Isoform 2 of SUN domain-containing ossification factor
Q8N4C7	Syntaxin-19
O14920;O14920-2;O14920-3;O14920-4	Inhibitor of nuclear factor kappa-B kinase subunit beta
Q9NPP4;Q9NPP4-2	NLR family CARD domain-containing protein 4
P23142-4	Isoform C of Fibulin-1
Q9UBB9;Q9UBB9-2	Tuftelin-interacting protein 11

Q70EL4-4	Isoform 4 of Ubiquitin carboxyl-terminal hydrolase 43
Q8N5G2;Q8N5G2-2	Macoilin
P02760	Protein AMBP
Q8TCU6;Q8TCU6-2;Q8TCU6-3	Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 protein
Q6NWY9-2;Q6NWY9;Q6NWY9-3	Isoform 2 of Pre-mRNA-processing factor 40 homolog B
P01023	Alpha-2-macroglobulin
Q86XR2-4;Q86XR2;Q86XR2-2;Q86XR2-3;Q86XR2-5	Isoform 4 of Niban-like protein 2
Q13224	Glutamate receptor ionotropic, NMDA 2B
I6L899;A6NCC3;A6NMD2;A6NP81;F8WBI6;H3BSY2;P0CJ92	Golgin subfamily A member 8R
Q5TCH4-2	Isoform 2 of Cytochrome P450 4A22
Q13562	Neurogenic differentiation factor 1
O75460	Serine/threonine-protein kinase/endoribonuclease IRE1
P19823	Inter-alpha-trypsin inhibitor heavy chain H2
Q15004	PCNA-associated factor
P57076	Uncharacterized protein C21orf59
P69905	Hemoglobin subunit alpha
Q7Z699	Sprouty-related, EVH1 domain-containing protein 1
P01617	Ig kappa chain V-II region TEW
Q6QHK4	Factor in the germline alpha
P68402-3;P68402;P68402-2;P68402-4	Isoform 3 of Platelet-activating factor acetylhydrolase IB subunit beta
A2RUB6;A2RUB6-2;A2RUB6-3;A2RUB6-4	Coiled-coil domain-containing protein 66
Q16787	Laminin subunit alpha-3
Q5VST9;Q13158;Q5VST9-2;Q5VST9-3	Obscurin
Q12791;Q12791-2;Q12791-3;Q12791-4;Q12791-5;Q12791-7	Calcium-activated potassium channel subunit alpha-1
P55055	Oxysterols receptor LXR-beta
P55056	Apolipoprotein C-IV
Q9HB07	UPF0160 protein MYG1, mitochondrial
Q6P587;Q6P587-2;Q6P587-3	Acylpyruvase FAHD1, mitochondrial
Q6IQ19	Centriole, cilia and spindle-associated protein
Q92844	TRAF family member-associated NF-kappa-B activator
Q01524	Defensin-6
P46937-2;P46937;P46937-3;P46937-4	Isoform 2 of Yorkie homolog
O43426;O43426-2;O43426-4;O43426-5	Synaptojanin-1
P01719	Ig lambda chain V-V region DEL
Q9NW15-3	Isoform 3 of Anoctamin-10
P05937	Calbindin
Q12768	WASH complex subunit strumpellin
Q9UKJ3;Q9UKJ3-2	G patch domain-containing protein 8
P59047	NACHT, LRR and PYD domains-containing protein 5
Q99871-3;Q99871;Q99871-2	Isoform 3 of HAUS augmin-like complex subunit 7
Q5VWP3;Q5VWP3-2;Q5VWP3-3	Muscular LMNA-interacting protein
Q9ULD0-2;Q9ULD0;Q9ULD0-3	Isoform 2 of 2-oxoglutarate dehydrogenase-like, mitochondrial
O95267;O95267-2;O95267-3;O95267-4;O95267-5	RAS guanyl-releasing protein 1
O75223	Gamma-glutamylcyclotransferase
P10909	Clusterin
P10909-2;P10909-4	Isoform 2 of Clusterin

P10909-5	Isoform 5 of Clusterin
Q9NXL2-2;Q9NXL2	Isoform 2 of Rho guanine nucleotide exchange factor 38
Q13363-2;Q13363	Isoform 2 of C-terminal-binding protein 1
Q8IWZ6-2;Q8IWZ6	Isoform 2 of Bardet-Biedl syndrome 7 protein
Q13029;Q13029-2;Q13029-3	PR domain zinc finger protein 2
O94818;O94818-3;O94818-4;Q4JDL3-12	Nucleolar protein 4
Q969I3	Glycine N-acyltransferase-like protein 1
Q9BX97	Plasmalemma vesicle-associated protein
Q4L180-2;Q4L180	Isoform 2 of Filamin A-interacting protein 1-like
Q9NUV9	GTPase IMAP family member 4
Q7Z7C7	Stimulated by retinoic acid gene 8 protein homolog
Q9Y2K9	Syntaxin-binding protein 5-like
Q06033;Q06033-2	Inter-alpha-trypsin inhibitor heavy chain H3
A6NI56-2;A6NI56	Isoform 2 of Coiled-coil domain-containing protein 154
P20337	Ras-related protein Rab-3B
Q6P1J6-3;Q6P1J6	Isoform 3 of Phospholipase B1, membrane-associated
Q6ZU52;Q6ZU52-2	Uncharacterized protein KIAA0408
Q9Y6Q6	Tumor necrosis factor receptor superfamily member 11A
Q9H5J0	Zinc finger and BTB domain-containing protein 3
A2PYH4;A2PYH4-2	Probable ATP-dependent DNA helicase HFM1
Q5TAA0;Q5TAA0-2	Tetratricopeptide repeat protein 22
Q6ZSZ6;Q6ZSZ6-2	Teashirt homolog 1
P80192;P80192-4	Mitogen-activated protein kinase kinase kinase 9
P80419	Ig heavy chain V-III region GAR
Q8TAQ9;Q8TAQ9-2	SUN domain-containing protein 3
P08709;P08709-2	Coagulation factor VII
P0C1S8	Wee1-like protein kinase 2
P42695	Condensin-2 complex subunit D3
P01861	Ig gamma-4 chain C region
Q9P2K8-2;Q9P2K8;Q9P2K8-3	Isoform 2 of Eukaryotic translation initiation factor 2-alpha kinase 4
Q6ZRP7	Sulfhydryl oxidase 2
Q53GA4	Pleckstrin homology-like domain family A member 2
A6NHI5	Transmembrane protein FLJ78588
Q58FF7	Putative heat shock protein HSP 90-beta-3
Q32P51	Heterogeneous nuclear ribonucleoprotein A1-like 2
P0C875;P0C875-2	Protein FAM228B
P26012	Integrin beta-8
Q8WUY3-3;Q8WUY3;Q8WUY3-2	Isoform 3 of Protein prune homolog 2
P00451	Coagulation factor VIII
Q99828	Calcium and integrin-binding protein 1
O95817	BAG family molecular chaperone regulator 3
P49454	Centromere protein F
O14791;O14791-2;O14791-3	Apolipoprotein L1
Q53EQ6-2;Q53EQ6	Isoform 2 of Tigger transposable element-derived protein 5
A8K5M9	Uncharacterized protein C15orf62, mitochondrial
P00734	Prothrombin
O95248-4;O95248	Isoform 4 of Myotubularin-related protein 5

Q9BW66-3;Q9BW66;Q9BW66-2	Isoform 3 of Cyclin-dependent kinase 2-interacting protein
Q8N8N7	Prostaglandin reductase 2
Q92830;Q92830-2	Histone acetyltransferase KAT2A
P22792	Carboxypeptidase N subunit 2
Q5T0F9-5	Isoform 5 of Coiled-coil and C2 domain-containing protein 1B
Q8IZS5;Q8IZS5-2;Q8IZS5-3;Q8IZS5-4;Q8IZS5-5	Orofacial cleft 1 candidate gene 1 protein
P49588	Alanine--tRNA ligase, cytoplasmic
Q14061	Cytochrome c oxidase copper chaperone
Q8IYU2;Q8IYU2-4	E3 ubiquitin-protein ligase HACE1
Q8NA19;Q8NA19-2	Lethal(3)malignant brain tumor-like protein 4
Q9H497;Q9H497-2	Torsin-3A
A6NDI0	Putative tripartite motif-containing protein 49B
Q9UJ14;Q9UJ14-5	Gamma-glutamyltransferase 7
Q6ZRI0	Otogelin
Q5EBL4	RILP-like protein 1
P49796-6;P49796;P49796-1;P49796-2;P49796-4;P49796-7	Isoform 6 of Regulator of G-protein signaling 3
Q9H1L0	Uncharacterized protein C20orf166
P01707	Ig lambda chain V-II region TRO
P78386	Keratin, type II cuticular Hb5
P42330	Aldo-keto reductase family 1 member C3
Q96F07-2;Q96F07	Isoform 2 of Cytoplasmic FMR1-interacting protein 2
Q8IWS0-4;Q8IWS0-2	Isoform 4 of PHD finger protein 6
P04208	Ig lambda chain V-I region WAH
P04275	von Willebrand factor
P00742	Coagulation factor X
P01702	Ig lambda chain V-I region NIG-64
P01909	HLA class II histocompatibility antigen, DQ alpha 1 chain
Q5T036	Putative FAM120A opposite strand protein
Q2M2E3	Outer dense fiber protein 4
P00747;Q6ZUS5;Q6ZUS5-2	Plasminogen
Q9HCD6;Q9HCD6-2	Protein TANC2
Q96PD5;Q96PD5-2	N-acetylmuramoyl-L-alanine amidase
Q8NCM8;P0CW27;Q86SR1;Q86SR1-2;Q8IUA7;Q8IUA7-3;Q8IUA7-4;Q8N139-2;Q8NCM8-2;Q8TET4	Cytoplasmic dynein 2 heavy chain 1
P31276	Homeobox protein Hox-C13
P01871;P01871-2	Ig mu chain C region
Q96KN9	Gap junction delta-4 protein
Q9Y5N6	Origin recognition complex subunit 6
Q02410	Amyloid beta A4 precursor protein-binding family A member 1
O60522;O60522-2	Tudor domain-containing protein 6
O14948-2;O14948	Isoform 2 of Transcription factor EC
Q9Y2Y8	Proteoglycan 3
P06681-2	Isoform 2 of Complement C2
Q5VX52	Spermatogenesis-associated protein 1
Q8IXH7;Q8IXH7-3;Q8IXH7-4	Negative elongation factor C/D
Q9NRX4-2	Isoform 2 of 14 kDa phosphohistidine phosphatase

Q7Z2W7	Transient receptor potential cation channel subfamily M member 8
O60784-2;O60784;O60784-3;O60784-4	Isoform 2 of Target of Myb protein 1
P51636-3	Isoform C of Caveolin-2
O15247	Chloride intracellular channel protein 2
O43790;P78385;Q14533	Keratin, type II cuticular Hb6
P01042-2	Isoform LMW of Kininogen-1
O00187-2	Isoform 2 of Mannan-binding lectin serine protease 2
Q8NI08-2;Q8NI08	Isoform 2 of Nuclear receptor coactivator 7
Q8NF91-8	Isoform 8 of Nesprin-1
Q494X1;Q494X1-2	Protein FAM153C
Q86Y22;Q86Y22-2	Collagen alpha-1(XXIII) chain
P01622	Ig kappa chain V-III region Ti
P23497	Nuclear autoantigen Sp-100
Q6P2H3;Q6P2H3-2	Centrosomal protein of 85 kDa
P62987	Ubiquitin-60S ribosomal protein L40
Q96BJ3-2;Q96BJ3	Isoform 2 of Axin interactor, dorsalization-associated protein
O43318	Mitogen-activated protein kinase kinase kinase 7
Q9GZP9	Derlin-2
Q8N3E9	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-3
Q14246-2;Q14246;Q14246-4;Q14246-5	Isoform 2 of EGF-like module-containing mucin-like hormone receptor-like 1
Q6ZV77	Uncharacterized protein C9orf139
Q8IW40	Coiled-coil domain-containing protein 103
P68106-2	Isoform 2 of Peptidyl-prolyl cis-trans isomerase FKBP1B
P01599	Ig kappa chain V-I region Gal
Q9P209-2;Q9P209	Isoform 2 of Centrosomal protein of 72 kDa
Q8WUW1-2;Q8WUW1	Isoform 2 of Protein BRICK1
Q16667-2	Isoform 2 of Cyclin-dependent kinase inhibitor 3
Q9UKT9;Q9UKT9-13;Q9UKT9-14;Q9UKT9-15;Q9UKT9-2;Q9UKT9-3;Q9UKT9-4;Q9UKT9-5;Q9UKT9-6;Q9UKT9-7;Q9UKT9-8	Zinc finger protein Aiolos
O95900	Probable tRNA pseudouridine synthase 2
A1L4Q6	Putative uncharacterized protein FLJ41423
Q9NY84-7;Q9NY84-8	Isoform 7 of Vascular non-inflammatory molecule 3
Q14192	Four and a half LIM domains protein 2
P00749	Urokinase-type plasminogen activator
Q6ZPA2	Putative uncharacterized protein FLJ26174
P13349	Myogenic factor 5
P55211-3;P55211;P55211-2	Isoform 3 of Caspase-9
Q9NZS9	Bifunctional apoptosis regulator
Q9C010;Q9C010-2	cAMP-dependent protein kinase inhibitor beta
Q9H7B7	Uncharacterized protein C7orf69
Q96EF6	F-box only protein 17
Q9UPQ4;Q9UPQ4-2	Tripartite motif-containing protein 35
P18136	Ig kappa chain V-III region HIC

B0I1T2-2	Isoform 2 of Unconventional myosin-Ig
Q15042;Q15042-2	Rab3 GTPase-activating protein catalytic subunit
Q99611	Selenide, water dikinase 2
Q92782-3;Q92782;Q92782-2	Isoform 3 of Zinc finger protein neuro-d4
P61925	cAMP-dependent protein kinase inhibitor alpha
Q8NG50;Q8NG50-10;Q8NG50-12;Q8NG50-3;Q8NG50-5;Q8NG50-7;Q8NG50-8	RAD52 motif-containing protein 1
Q53EL6;Q53EL6-2	Programmed cell death protein 4
Q7RTS7	Keratin, type II cytoskeletal 74
P48509	CD151 antigen
Q8WZ33	MaFF-interacting protein
Q9NX14;Q9NX14-2	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial
Q6UB99	Ankyrin repeat domain-containing protein 11
Q7Z7M8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8
Q9UBA6	Protein G8
Q6UW63	KDEL motif-containing protein 1
Q9NPE2-3	Isoform 2 of Neugrin
P82909	28S ribosomal protein S36, mitochondrial
P16083	Ribosyldihydronicotinamide dehydrogenase [quinone]
O75822	Eukaryotic translation initiation factor 3 subunit J
Q86UA6-8	Isoform 8 of RPA-interacting protein
Q8ND83	SLAIN motif-containing protein 1
P31275	Homeobox protein Hox-C12
Q86YR5;Q86YR5-2;Q86YR5-4	G-protein-signaling modulator 1
A0AVI2	Fer-1-like protein 5
Q96FQ7	Putative uncharacterized protein encoded LINC00526
Q9NRX2	39S ribosomal protein L17, mitochondrial
Q8N819;Q8N819-3	Probable protein phosphatase 1N
Q7Z449;Q7Z449-2	Cytochrome P450 2U1
Q5BKZ1;Q5BKZ1-2	DBIRD complex subunit ZNF326
O43825	Beta-1,3-galactosyltransferase 2
P67936-2	Isoform 2 of Tropomyosin alpha-4 chain
Q8TAY7	Protein FAM110D
P58557-3;P58557	Isoform C of Putative ribonuclease
A2IDD5	Coiled-coil domain-containing protein 78
P35244	Replication protein A 14 kDa subunit
P13639	Elongation factor 2
Q5TFE4	5'-nucleotidase domain-containing protein 1
Q99471	Prefoldin subunit 5
Q13748	Tubulin alpha-3C/D chain
Q8NFV4;Q8NFV4-2;Q8NFV4-3;Q8NFV4-4;Q8NFV4-5;Q8NFV4-6	Alpha/beta hydrolase domain-containing protein 11
Q7L8A9	Vasohibin-1
Q9UNP4;Q9UNP4-2;Q9UNP4-3	Lactosylceramide alpha-2,3-sialyltransferase

O43687	A-kinase anchor protein 7 isoforms alpha and beta
Q8NBI6	Xyloside xylosyltransferase 1
Q9H9P8-2;Q9H9P8	Isoform 2 of L-2-hydroxyglutarate dehydrogenase, mitochondrial
P78563-4	Isoform 4 of Double-stranded RNA-specific editase 1
Q9Y6I7;Q9Y6I7-3	WD repeat and SOCS box-containing protein 1
P55822;P55822-2	SH3 domain-binding glutamic acid-rich protein
P07202-7;P07202;P07202-2;P07202-3;P07202-4;P07202-8	Isoform 2-3 of Thyroid peroxidase
P46779-4;P46779;P46779-2;P46779-3;P46779-5	Isoform 4 of 60S ribosomal protein L28
Q04759-2	Isoform 2 of Protein kinase C theta type
Q5FWF4	DNA annealing helicase and endonuclease ZRANB3
Q9NQW5	Probable histone-lysine N-methyltransferase PRDM7
Q8WWF8	Calcyphosin-like protein
O96001-2;O96001	Isoform 2 of Protein phosphatase 1 regulatory subunit 17
Q9UJ94	Putative uncharacterized protein encoded C21orf104
Q9P000	COMM domain-containing protein 9
Q86TJ2;Q86TJ2-2;Q86TJ2-3	Transcriptional adapter 2-beta
Q9P0W2-3;Q9P0W2;Q9P0W2-2	Isoform 3 of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1-related
P04406-2;P04406	Isoform 2 of Glyceraldehyde-3-phosphate dehydrogenase
P27816-7	Isoform 7 of Microtubule-associated protein 4
Q9H254;Q9H254-2;Q9H254-4	Spectrin beta chain, non-erythrocytic 4
Q5VXD3-4	Isoform 4 of Sterile alpha motif domain-containing protein 13
E5RG02	Putative serine protease 46
Q9HBQ8;Q9HBQ8-2	Putative golgin subfamily A member 2B
O95628-3;O95628	Isoform 3 of CCR4-NOT transcription complex subunit 4
P25963	NF-kappa-B inhibitor alpha
Q9NR81	Rho guanine nucleotide exchange factor 3
Q96PX6	Coiled-coil domain-containing protein 85A
Q6P3W2	DnaJ homolog subfamily C member 24
Q86US8	Telomerase-binding protein EST1A
A8MWD9	Small nuclear ribonucleoprotein G-like protein
O95183	Vesicle-associated membrane protein 5
Q16623-2	Isoform 2 of Syntaxin-1A
Q8TF72-2	Isoform 2 of Protein Shroom3
P49767	Vascular endothelial growth factor C
Q6ZRY4	RNA-binding protein with multiple splicing 2
P60903	Protein S100-A10
Q9BV44	THUMP domain-containing protein 3
Q96IX9	Putative ankyrin repeat domain-containing protein 26-like 1
Q9Y6I7-2	Isoform 2 of WD repeat and SOCS box-containing protein 1
Q96DH6;Q96DH6-2;Q96DH6-3	RNA-binding protein Musashi homolog 2
Q5VVJ2;Q5VVJ2-2;Q5VVJ2-3	Histone H2A deubiquitinase MYSM1
P09455;P09455-2;P09455-3	Retinol-binding protein 1
Q9H714;Q9H714-3;Q9H714-4	Uncharacterized protein KIAA0226-like

O60936	Nucleolar protein 3
Q8NC56-2	Isoform 2 of LEM domain-containing protein 2
O75410-3;O75410;O75410-2;O75410-4;O75410-5;O75410-6	Isoform 3 of Transforming acidic coiled-coil-containing protein 1
Q9UHC6;Q8NFK1	Contactin-associated protein-like 2
Q16363-2;Q16363	Isoform 2 of Laminin subunit alpha-4
Q12769;Q12769-2	Nuclear pore complex protein Nup160
Q8IW70	Transmembrane protein 151B
Q9NUL3-8;Q9NUL3;Q9NUL3-2;Q9NUL3-3	Isoform 8 of Double-stranded RNA-binding protein Staufen homolog 2
Q00059-2;Q00059	Isoform 2 of Transcription factor A, mitochondrial
Q8TD17;Q8TD17-2	Zinc finger protein 398
O15037	Protein KHNYN
P20823	Hepatocyte nuclear factor 1-alpha
Q9BV19	Uncharacterized protein C1orf50
Q8NEQ6	Uncharacterized protein C1orf64
Q6UW60;Q6UW60-2	Proprotein convertase subtilisin/kexin type 4
Q15004-2	Isoform 2 of PCNA-associated factor
Q9C0C6	Uncharacterized protein KIAA1737
Q8N4V1-2	Isoform 2 of Membrane magnesium transporter 1
O43852;O43852-2;O43852-3;O43852-4	Calumenin
A2RRD8	Zinc finger protein 320
O60828-8	Isoform 8 of Polyglutamine-binding protein 1
O75083;O75083-3	WD repeat-containing protein 1
P06309	Ig kappa chain V-II region GM607 (Fragment)
Q9BSG5-2	Isoform 2 of Retbindin
Q9UIR0;Q9UIR0-2;Q9UIR0-4;Q9UIR0-5	Butyrophilin-like protein 2
A4FU69;A4FU69-2;A4FU69-3;A4FU69-4	EF-hand calcium-binding domain-containing protein 5
Q5FWF6	Zinc finger protein 789
P43080	Guanylyl cyclase-activating protein 1
Q6ZUX3;Q6ZUX3-2	Protein FAM179A
O15315-1	Isoform 2 of DNA repair protein RAD51 homolog 2
Q9BPZ7-6;Q9BPZ7;Q9BPZ7-3	Isoform 6 of Target of rapamycin complex 2 subunit MAPKAP1
P61568	HERV-K_1p13.3 provirus ancestral Env polyprotein
P07585-5	Isoform E of Decorin
Q32NC0	UPF0711 protein C18orf21
Q9NV06-2	Isoform 2 of DDB1- and CUL4-associated factor 13
P0CE72	Oncomodulin-1
P21980-3	Isoform 3 of Protein-glutamine gamma-glutamyltransferase 2
A8K554	Putative protein ZNF815
Q6ZUT6-3;Q6ZUT6-4	Isoform 3 of Uncharacterized protein C15orf52
Q8WV48-4	Isoform 4 of Coiled-coil domain-containing protein 107
P08311	Cathepsin G
Q15369	Transcription elongation factor B polypeptide 1
Q6ZS52	Putative uncharacterized protein FLJ45825

Q96F85-2	Isoform 2 of CB1 cannabinoid receptor-interacting protein 1
O15467	C-C motif chemokine 16
Q8N8A8	Protein FAM169B
O43678	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2
Q99715-4;Q99715;Q99715-2	Isoform 4 of Collagen alpha-1(XII) chain
Q49B96	Cytochrome c oxidase assembly protein COX19
Q9HCH0-4	Isoform 4 of Nck-associated protein 5-like
Q6IQ19-4	Isoform 4 of Centriole, cilia and spindle-associated protein
Q8NDC4-2	Isoform 2 of MORN repeat-containing protein 4
P00480	Ornithine carbamoyltransferase, mitochondrial
Q9BZS1-2;Q9BZS1;Q9BZS1-3;Q9BZS1-4	Isoform 2 of Forkhead box protein P3
P11233	Ras-related protein Ral-A
Q9Y3C5	RING finger protein 11
Q5MJ09	Sperm protein associated with the nucleus on the X chromosome N3
Q96AX2-4;Q96AX2;Q96AX2-2;Q96AX2-3	Isoform 4 of Ras-related protein Rab-37
Q9H3Y8	Pancreatic progenitor cell differentiation and proliferation factor
Q9C004	Protein sprouty homolog 4
Q8IXP5	Uncharacterized protein C11orf53
Q9BSY4	Coiled-coil-helix-coiled-coil-helix domain-containing protein 5
Q9UBX0	Homeobox expressed in ES cells 1
O14603-2	Isoform 2 of PTPN13-like protein, Y-linked
Q9Y478	5'-AMP-activated protein kinase subunit beta-1
Q5JUW0-3	Isoform 3 of KRAB domain-containing protein 4
Q9UK76-3	Isoform 3 of Hematological and neurological expressed 1 protein
A6NML5	Transmembrane protein 212
A2RUC4	tRNA wybutosine-synthesizing protein 5
Q93063;Q93063-2;Q93063-3	Exostosin-2
Q9NP73-2	Isoform 2 of Putative bifunctional UDP-N-acetylglucosamine transferase and deubiquitinase ALG13
O60245;O60245-2	Protocadherin-7
Q15020	Squamous cell carcinoma antigen recognized by T-cells 3
Q9BVI0-2	Isoform 2 of PHD finger protein 20
Q5GLZ8-5	Isoform 5 of Probable E3 ubiquitin-protein ligase HERC4
Q86T26	Transmembrane protease serine 11B
Q9H3K6	BolA-like protein 2
Q6ZNB6;Q6ZNB6-2	NF-X1-type zinc finger protein NFXL1
Q02325	Plasminogen-like protein B
P55773-2	Isoform Long of C-C motif chemokine 23
P01588	Erythropoietin
P01891	HLA class I histocompatibility antigen, A-68 alpha chain
Q5VSR9	Sperm protein associated with the nucleus on the X chromosome N1
P10316	HLA class I histocompatibility antigen, A-69 alpha chain
O15525	Transcription factor MafG
P01892	HLA class I histocompatibility antigen, A-2 alpha chain

A1L188	Uncharacterized protein C17orf89
Q8TDM5	Sperm acrosome membrane-associated protein 4
P36543-3	Isoform 3 of V-type proton ATPase subunit E 1
Q9UNW1-4;Q9UNW1	Isoform 4 of Multiple inositol polyphosphate phosphatase 1
P07741	Adenine phosphoribosyltransferase
O15273	Telethonin
Q7Z434-5	Isoform 5 of Mitochondrial antiviral-signaling protein
Q9HC29-2;Q9HC29;Q9HC29-3	Isoform 2 of Nucleotide-binding oligomerization domain-containing protein 2
Q9NVL1-2;Q9NVL1;Q9NVL1-3	Isoform 2 of Protein FAM86C1
Q6WN34-3	Isoform 3 of Chordin-like protein 2
Q8WVD5	RING finger protein 141
O14746;O14746-2;O14746-3;O14746-4	Telomerase reverse transcriptase
Q969T3	Sorting nexin-21
P34096	Ribonuclease 4
P07333;P07333-2	Macrophage colony-stimulating factor 1 receptor
Q9Y6I9	Testis-expressed sequence 264 protein
P06316	Ig lambda chain V-I region BL2
Q6UWY2	Serine protease 57
Q13099;Q13099-2;Q13099-3	Intraflagellar transport protein 88 homolog
P02751-10;P02751-13	Isoform 10 of Fibronectin
Q9UMF0	Intercellular adhesion molecule 5
P20338	Ras-related protein Rab-4A
Q8NEG2-2	Isoform 2 of Uncharacterized protein C7orf57
Q8N365-2	Isoform 2 of Uncharacterized protein C1orf51
Q5THR3;Q5THR3-2;Q5THR3-5	EF-hand calcium-binding domain-containing protein 6
O75368	SH3 domain-binding glutamic acid-rich-like protein
Q96FE7	Phosphoinositide-3-kinase-interacting protein 1
Q00987-4	Isoform Mdm2-B of E3 ubiquitin-protein ligase Mdm2
Q86U37	Uncharacterized protein C14orf23
P0CW21	Putative uncharacterized protein SPG20OS
Q6IB77-2;Q6IB77	Isoform 2 of Glycine N-acyltransferase
P57086	SCAN domain-containing protein 1
P53672	Beta-crystallin A2
Q12841	Follistatin-related protein 1
Q5JWF8	Actin-like protein 10
Q5T197;Q5T197-2;Q5T197-3	DC-STAMP domain-containing protein 1
P35080	Profilin-2
Q14236	Early lymphoid activation gene protein
Q7Z3Z0	Keratin, type I cytoskeletal 25
Q969G6	Riboflavin kinase
Q9Y215-7	Isoform VII of Acetylcholinesterase collagenic tail peptide
Q86VE3-2;Q86VE3	Isoform 2 of Spermidine/spermine N(1)-acetyltransferase-like protein 1
Q9NTQ9	Gap junction beta-4 protein

Q8TDV0	Probable G-protein coupled receptor 151
Q5VVX9-2;Q5VVX9	Isoform 2 of Ubiquitin-conjugating enzyme E2 U
Q7Z2E3-11;Q7Z2E3-6;Q7Z2E3-9	Isoform 11 of Aprataxin
P78358-2	Isoform 2 of Cancer/testis antigen 1
Q9BV47	Dual specificity protein phosphatase 26
Q7Z2F6	Putative protein ZNF720
A8MUK1;C9J2P7;C9JLJ4;C9JVI0;D6R9N7;D6RA61;D6RBQ6;D6RCP7;D6RJB6;Q0WX57	Ubiquitin carboxyl-terminal hydrolase 17-like protein 5
Q8IW45-4	Isoform 4 of ATP-dependent (S)-NAD(P)H-hydrate dehydratase
Q9GIP4-1;Q8MH63;Q9GIP4	Isoform 2 of Putative L-type amino acid transporter 1-like protein IMAA
Q96Q80-4	Isoform 4 of Derlin-3
P14060;P26439;P26439-2	3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase type 1
Q03014	Hematopoietically-expressed homeobox protein HHEX
Q9NRF9	DNA polymerase epsilon subunit 3
Q9H628	Ras-related and estrogen-regulated growth inhibitor-like protein
P36639-2;P36639	Isoform p22 of 7,8-dihydro-8-oxoguanine triphosphatase
Q9P1U1	Actin-related protein 3B
P81534	Beta-defensin 103
Q9BY65	Nasopharyngeal carcinoma down-regulated gene protein 1
Q8WWF6	DnaJ homolog subfamily B member 3
P63218	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-5
Q3SX64	Outer dense fiber protein 3-like protein 2
Q969Q6-2	Isoform 2 of Serine/threonine-protein phosphatase 2A regulatory subunit B" subunit gamma
P14927	Cytochrome b-c1 complex subunit 7
P50135-3;P50135;P50135-2	Isoform 3 of Histamine N-methyltransferase
Q9UET6-2	Isoform 2 of Putative tRNA (cytidine(32)/guanosine(34)-2'-O)-methyltransferase
Q8IZN7	Beta-defensin 107
Q8IUE0	Homeobox protein TGIF2LY
Q96IF1	LIM domain-containing protein ajuba
Q9UKV3-2	Isoform 2 of Apoptotic chromatin condensation inducer in the nucleus
P42771-3	Isoform 3 of Cyclin-dependent kinase inhibitor 2A, isoforms 1/2/3
Q8WVF5	BTB/POZ domain-containing protein KCTD4
Q5SRH9-5;Q5SRH9;Q5SRH9-4	Isoform 5 of Tetratricopeptide repeat protein 39A
Q92876-2;Q92876	Isoform 2 of Kallikrein-6
Q03060-9;Q03060-11	Isoform 7 of cAMP-responsive element modulator
Q9BXJ8-2;Q9BXJ8	Isoform 2 of Transmembrane protein 120A
Q9HCE0;Q9HCE0-2	Ectopic P granules protein 5 homolog
Q6ZVN8	Hemojuvelin
Q5TZA2;Q5TZA2-2	Rootletin
Q9UKW6-3;Q9UKW6;Q9UKW6-2	Isoform 3 of ETS-related transcription factor Elf-5
Q5JPI9	Methyltransferase-like protein 10
P81877-4;P81877;P81877-3;P81877-5	Isoform 4 of Single-stranded DNA-binding protein 2
A6NEV1	Proline-rich protein 23A

Q3V6T2;Q3V6T2-2;Q3V6T2-3;Q3V6T2-4;Q3V6T2-5	Girdin
Q9Y2B9	cAMP-dependent protein kinase inhibitor gamma
P01610	Ig kappa chain V-I region WEA
Q15417	Calponin-3
Q8WZ64	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 2
Q9NVV5-5;Q9NVV5;Q9NVV5-2;Q9NVV5-3;Q9NVV5-4	Isoform 5 of Androgen-induced gene 1 protein
Q5FWF4-3;Q5FWF4-2	Isoform 3 of DNA annealing helicase and endonuclease ZRANB3
Q6P9F5-2;Q6P9F5	Isoform 2 of Tripartite motif-containing protein 40
Q99584	Protein S100-A13
Q96EX2-4;Q96EX2	Isoform 4 of RING finger and transmembrane domain-containing protein 2
P41236;Q6NXS1	Protein phosphatase inhibitor 2
Q7Z4S9	SH2 domain-containing protein 6
O60828-5	Isoform 5 of Polyglutamine-binding protein 1
A6NJ69	IgA-inducing protein homolog
Q629K1	Triple QxxK/R motif-containing protein
Q8WUX2	Cation transport regulator-like protein 2
Q5QGZ9-4;Q5QGZ9	Isoform 4 of C-type lectin domain family 12 member A
Q9BXU8	Ferritin heavy polypeptide-like 17
P22570-4;P22570;P22570-2;P22570-3;P22570-5;P22570-6;P22570-7	Isoform 4 of NADPH:adrenodoxin oxidoreductase, mitochondrial
P59103-4;P59103	Isoform 4 of D-amino acid oxidase activator
Q8IY67-3	Isoform 3 of Ribonucleoprotein PTB-binding 1
Q9BXN2-5;Q9BXN2;Q9BXN2-2	Isoform 5 of C-type lectin domain family 7 member A
Q9NZI2	Kv channel-interacting protein 1
Q9P0M2	A-kinase anchor protein 7 isoform gamma
Q5TBK1	NEDD4-binding protein 2-like 1
Q9BZG9-3	Isoform 3 of Ly-6/neurotoxin-like protein 1
Q9H008-2;Q9H008	Isoform 2 of Phospholysine phosphohistidine inorganic pyrophosphate phosphatase
P07108-2	Isoform 2 of Acyl-CoA-binding protein
Q9BV29;Q9BV29-2	Uncharacterized protein C15orf57
O95996;O95996-3	Adenomatous polyposis coli protein 2
Q6ZN30;Q6ZN30-2	Zinc finger protein basonuclin-2
Q4JDL3-2;Q4JDL3-11;Q4JDL3-15;Q4JDL3-8	Isoform 2 of Tyrosine-protein phosphatase non-receptor type 20
P19387	DNA-directed RNA polymerase II subunit RPB3
Q30KQ8	Beta-defensin 112
Q8TDH9;Q8TDH9-2;Q8TDH9-3	Biogenesis of lysosome-related organelles complex 1 subunit 5
Q9UDY6	Tripartite motif-containing protein 10
Q9HAU5	Regulator of nonsense transcripts 2
Q9Y259-2	Isoform 2 of Choline/ethanolamine kinase
O14713;O14713-2	Integrin beta-1-binding protein 1
P35680;P35680-3	Hepatocyte nuclear factor 1-beta
Q96CF2	Charged multivesicular body protein 4c
P35858-2	Isoform 2 of Insulin-like growth factor-binding protein complex acid labile subunit

P0C7V5	Putative zinc finger protein 812
Q9Y546	Leucine-rich repeat-containing protein 42
Q86YR6	POTE ankyrin domain family member D
Q9Y6K8;Q9Y6K8-2;Q9Y6K8-3	Adenylate kinase isoenzyme 5
P68363;Q13748-2;Q6PEY2;Q71U36-2;Q9BQE3	Tubulin alpha-1B chain
Q9NTI5;Q9NTI5-2	Sister chromatid cohesion protein PDS5 homolog B
O15068;O15068-10;O15068-4;O15068-6;O15068-8;O15068-9	Guanine nucleotide exchange factor DBS
P02042	Hemoglobin subunit delta
A8MQ03	UPF0574 protein C9orf169
O75771-8	Isoform 8 of DNA repair protein RAD51 homolog 4
O00628	Peroxisomal targeting signal 2 receptor
P06888	Ig lambda chain V-I region EPS
Q9H5N1	Rab GTPase-binding effector protein 2
Q6UXQ4	Uncharacterized protein C2orf66
Q96HQ2	CDKN2AIP N-terminal-like protein
P61457	Pterin-4-alpha-carbinolamine dehydratase
Q9H9P5	Putative E3 ubiquitin-protein ligase UNKL
Q9Y6K9;Q9Y6K9-2	NF-kappa-B essential modulator
Q99440	Uncharacterized protein C4orf6
Q9NQR7	Coiled-coil domain-containing protein 177
Q9NQ31;Q9NQ31-3	A-kinase-interacting protein 1
Q9UKU0-2	Isoform 2 of Long-chain-fatty-acid--CoA ligase 6
Q8N6N2-2	Isoform 2 of Tetratricopeptide repeat protein 9B
Q99956	Dual specificity protein phosphatase 9
Q9HBH0	Rho-related GTP-binding protein RhoF
P35858	Insulin-like growth factor-binding protein complex acid labile subunit
P30086	Phosphatidylethanolamine-binding protein 1
Q68CZ2;Q68CZ2-2;Q68CZ2-3;Q68CZ2-4	Tensin-3
P68133	Actin, alpha skeletal muscle
A6NL28	Putative tropomyosin alpha-3 chain-like protein
P04278-5	Isoform 5 of Sex hormone-binding globulin
Q86T96;Q86T96-2;Q86T96-3	E3 ubiquitin-protein ligase RNF180
P35250-2	Isoform 2 of Replication factor C subunit 2
A6BM72-4	Isoform 4 of Multiple epidermal growth factor-like domains protein 11
Q9Y6E0;Q9Y6E0-2	Serine/threonine-protein kinase 24
P68032	Actin, alpha cardiac muscle 1
Q13867	Bleomycin hydrolase
Q9NS26;Q9NY87	Sperm protein associated with the nucleus on the X chromosome A
A0M8Q6	Ig lambda-7 chain C region
Q9H8W5;Q9H8W5-2	Tripartite motif-containing protein 45
P06311	Ig kappa chain V-III region IARC/BL41
O75715	Epididymal secretory glutathione peroxidase
Q7L0Q8	Rho-related GTP-binding protein RhoU

Q86UK0-2	Isoform 2 of ATP-binding cassette sub-family A member 12
P12081-3;P12081;P12081-2;P12081-4	Isoform 3 of Histidine--tRNA ligase, cytoplasmic
Q86YB7-2;Q86YB7	Isoform 2 of Enoyl-CoA hydratase domain-containing protein 2, mitochondrial
O00213-3;O00213;O00213-2;O00213-4	Isoform 3 of Amyloid beta A4 precursor protein-binding family B member 1
P01611	Ig kappa chain V-I region Wes
Q8TD10;Q8TD10-3	Mirror-image polydactyly gene 1 protein
Q9NRP4	Protein ACN9 homolog, mitochondrial
P01824	Ig heavy chain V-II region WAH
Q99457	Nucleosome assembly protein 1-like 3
Q8N4C8;Q8N4C8-4	Misshapen-like kinase 1
Q8NAV2-2;Q8NAV2	Isoform 2 of Uncharacterized protein C8orf58
Q8N8N0	E3 ubiquitin-protein ligase RNF152
P60900	Proteasome subunit alpha type-6
Q8N4C8-2;Q8N4C8-3;Q8N4C8-5	Isoform 1 of Misshapen-like kinase 1
P02458;P02458-1;P02458-3	Collagen alpha-1(II) chain
Q86VH2;Q86VH2-3	Kinesin-like protein KIF27
Q9C029	Tripartite motif-containing protein 7
Q8TDS7	Mas-related G-protein coupled receptor member D
P28482;P28482-2	Mitogen-activated protein kinase 1
Q6ZRV2	Protein FAM83H
P0C7V0	Putative uncharacterized protein encoded by LINC00271
Q9H3U1;Q9H3U1-2	Protein unc-45 homolog A
Q9UJ78;Q9UJ78-1;Q9UJ78-2	Zinc finger MYM-type protein 5
P55316	Forkhead box protein G1
P49411	Elongation factor Tu, mitochondrial
Q99592-2;Q99592	Isoform 2 of Zinc finger and BTB domain-containing protein 18
Q9NVZ3;Q9NVZ3-4	Adaptin ear-binding coat-associated protein 2
A4FU69-5	Isoform 5 of EF-hand calcium-binding domain-containing protein 5
Q9ULT0;Q9ULT0-3	Tetratricopeptide repeat protein 7A
Q8NBT2	Kinetochore protein Spc24
P60484	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN
O43692	Peptidase inhibitor 15
P18463	HLA class I histocompatibility antigen, B-37 alpha chain
Q96CM4	Nucleoredoxin-like protein 1
Q9H9P5-5;Q9H9P5-6	Isoform 5 of Putative E3 ubiquitin-protein ligase UNKL
P36915;P36915-2	Guanine nucleotide-binding protein-like 1
Q96M29	Tektin-5
A8MWE9	EF-hand calcium-binding domain-containing protein 8
Q02218	2-oxoglutarate dehydrogenase, mitochondrial
Q07812-6;Q07812;Q07812-2;Q07812-4;Q07812-5;Q07812-7;Q07812-8	Isoform Zeta of Apoptosis regulator BAX
Q8NDH6	Islet cell autoantigen 1-like protein
P01210	Proenkephalin-A
Q5JPB2	Zinc finger protein 831

Q13126-7;Q13126-4;Q13126-5;Q13126-6	Isoform 7 of S-methyl-5'-thioadenosine phosphorylase
P14920	D-amino-acid oxidase
Q6IPT4-4;Q6IPT4	Isoform 3 of NADH-cytochrome b5 reductase-like
Q8WVT3	Trafficking protein particle complex subunit 12
P04217-2	Isoform 2 of Alpha-1B-glycoprotein
Q9HBT8	Zinc finger protein 286A
Q96RG2;Q96RG2-2	PAS domain-containing serine/threonine-protein kinase
A2A288;A2A288-3;A2A288-4	Probable ribonuclease ZC3H12D
Q9P2M1-2;Q9P2M1	Isoform 2 of LRP2-binding protein
Q9BRR0;Q9BRR0-2	Zinc finger protein with KRAB and SCAN domains 3
Q86TP1-5;Q86TP1;Q86TP1-2;Q86TP1-3;Q86TP1-4;Q86TP1-6;Q86TP1-7	Isoform 5 of Protein prune homolog
Q9NRN5-3	Isoform 3 of Olfactomedin-like protein 3
Q86YJ6;Q86YJ6-2;Q86YJ6-3;Q86YJ6-4	Threonine synthase-like 2
Q8NE28	Probable inactive protein kinase-like protein SgK071
Q06495-2	Isoform 2 of Sodium-dependent phosphate transport protein 2A
Q9H1K0	Rabenosyn-5
Q9UNM6;Q9UNM6-2	26S proteasome non-ATPase regulatory subunit 13
Q9Y6U3;Q9Y6U3-2	Adseverin
Q86TY3-2;Q86TY3	Isoform 2 of Uncharacterized protein C14orf37
O95155-3	Isoform 3 of Ubiquitin conjugation factor E4 B
P55058-2	Isoform 2 of Phospholipid transfer protein
Q3MJ40;Q3MJ40-2;Q6NUI1	Coiled-coil domain-containing protein 144B
Q6NSJ2;Q6NSJ2-2	Pleckstrin homology-like domain family B member 3
P00915	Carbonic anhydrase 1
A6NLW8	Double homeobox protein A
P06313;P06312;P06314	Ig kappa chain V-IV region JI
Q6ZS81	WD repeat- and FYVE domain-containing protein 4
Q9H9H5	MAP6 domain-containing protein 1
Q8ND90	Paraneoplastic antigen Ma1
O15519;O15519-10;O15519-11;O15519-12;O15519-13;O15519-14;O15519-2;O15519-5;O15519-8;O15519-9	CASP8 and FADD-like apoptosis regulator
Q9H0W7	THAP domain-containing protein 2
Q5VWQ0	Round spermatid basic protein 1
Q02779	Mitogen-activated protein kinase kinase kinase 10
P78563-2;P78563;P78563-3	Isoform 2 of Double-stranded RNA-specific editase 1
Q6ZNM6	Uncharacterized protein C5orf48
P23942	Peripherin-2
Q6PJI9;Q6PJI9-2;Q6PJI9-3;Q6PJI9-4	WD repeat-containing protein 59
Q8TE57;Q8TE57-2	A disintegrin and metalloproteinase with thrombospondin motifs 16
P43220	Glucagon-like peptide 1 receptor
Q9C0E8-3;Q9C0E8;Q9C0E8-2	Isoform 3 of Protein lunapark
Q96DY2-2	Isoform 2 of IQ domain-containing protein D
Q6UXH1;Q6UXH1-2;Q6UXH1-3;Q6UXH1-4;Q6UXH1-5;Q6UXH1-6	Cysteine-rich with EGF-like domain protein 2

Q8IX18-2;Q8IX18	Isoform 2 of Probable ATP-dependent RNA helicase DHX40
Q6PD62	RNA polymerase-associated protein CTR9 homolog
Q8IXS6	Paralemmin-2
Q9BPY8-3;Q9BPY8-4	Isoform 3 of Homeodomain-only protein
Q6PGQ1	Uncharacterized protein C22orf43
P62166	Neuronal calcium sensor 1
P55196-5;P55196;P55196-1;P55196-2;P55196-3;P55196-6	Isoform 5 of Afadin
Q9Y5K3	Choline-phosphate cytidylyltransferase B
P04040	Catalase
Q92619;Q92619-2	Minor histocompatibility protein HA-1
Q8N139	ATP-binding cassette sub-family A member 6
Q9UH17	DNA dC->dU-editing enzyme APOBEC-3B
Q8TAB5	UPF0500 protein C1orf216
Q9UHD4	Cell death activator CIDE-B
Q96D46	60S ribosomal export protein NMD3
A0AVF1	Tetratricopeptide repeat protein 26
Q15506	Sperm surface protein Sp17
Q8IVE0	Putative ciliary rootlet coiled-coil protein-like 2 protein
O60928;O60928-2	Inward rectifier potassium channel 13
Q8WYJ6	Septin-1
Q96LC9-3	Isoform 3 of Bcl-2-modifying factor
P06239;P06239-2;P06239-3	Tyrosine-protein kinase Lck
O43772	Mitochondrial carnitine/acylcarnitine carrier protein
Q8N5P1	Zinc finger CCCH domain-containing protein 8
Q9NRC1	Suppressor of tumorigenicity 7 protein
P51114-2	Isoform 2 of Fragile X mental retardation syndrome-related protein 1
Q15940	Putative zinc finger protein 726P1
Q8IZT9	Protein FAM9C
Q9UIQ6-3;Q9UIQ6;Q9UIQ6-2	Isoform 3 of Leucyl-cystinyl aminopeptidase
Q9NZI2-3	Isoform 3 of Kv channel-interacting protein 1
Q9UHF5	Interleukin-17B
Q8TCC3-2	Isoform 2 of 39S ribosomal protein L30, mitochondrial
P29466;P29466-2;P29466-3	Caspase-1
Q96D42	Hepatitis A virus cellular receptor 1
Q8NCE8	Golgin subfamily A member 2-like protein 3
P57740	Nuclear pore complex protein Nup107
Q7Z2X7;Q5JRK9	P antigen family member 2
Q6UWW8;Q6UWW8-2	Carboxylesterase 3
Q9BZM5	NKG2D ligand 2
Q8N9Z0-2;Q8N9Z0	Isoform 2 of Zinc finger protein 610
Q6UWS5	Protein PET117 homolog, mitochondrial
P29508;P29508-2	Serpin B3
O60229-3	Isoform 3 of Kalirin

O15315-2	Isoform 3 of DNA repair protein RAD51 homolog 2
Q9GZZ1	N-alpha-acetyltransferase 50
Q4G0X9-2;Q4G0X9;Q4G0X9-4;Q4G0X9-5	Isoform 2 of Coiled-coil domain-containing protein 40
Q8NI36	WD repeat-containing protein 36
Q13398-2;Q13398-8	Isoform 2 of Zinc finger protein 211
Q92882	Osteoclast-stimulating factor 1
P23280;P23280-3	Carbonic anhydrase 6
P16949	Stathmin
Q14142-3;Q14142;Q14142-2	Isoform 3 of Tripartite motif-containing protein 14
Q96EE4	Coiled-coil domain-containing protein 126
Q7RTT3	Protein SSX9
O75830	Serpin I2
Q14994-10;Q14994;Q14994-11;Q14994-2;Q14994-3;Q14994-4;Q14994-5;Q14994-6;Q14994-7;Q14994-8;Q14994-9	Isoform 10 of Nuclear receptor subfamily 1 group I member 3
Q86WI3-4;Q86WI3-5	Isoform 4 of Protein NLRC5
Q9NUG6	p53 and DNA damage-regulated protein 1
K7EJ46-3;K7EJ46;K7EJ46-2	Isoform 3 of Small integral membrane protein 22
Q16670	Zinc finger and SCAN domain-containing protein 26
Q8IVS8-5;Q8IVS8;Q8IVS8-2;Q8IVS8-4;Q8IVS8-7	Isoform 5 of Glycerate kinase
Q9HCF6-11;Q9HCF6;Q9HCF6-2;Q9HCF6-3;Q9HCF6-4;Q9HCF6-5;Q9HCF6-6;Q9HCF6-7;Q9HCF6-8	Isoform 11 of Transient receptor potential cation channel subfamily M member 3
Q8IUE1	Homeobox protein TGIF2LX
O60488-2;O60488	Isoform Short of Long-chain-fatty-acid--CoA ligase 4
Q9H6Y2;Q9H6Y2-2	WD repeat-containing protein 55
Q8IZF5;Q8IZF5-3	Probable G-protein coupled receptor 113
Q96FZ7	Charged multivesicular body protein 6
Q92963;Q92963-2;Q92963-3	GTP-binding protein Rit1
P14921;P14921-2;P14921-3	Protein C-ets-1
Q5JSJ4	Protein DDX26B
Q86SZ2;Q86SZ2-2	Trafficking protein particle complex subunit 6B
A8MXQ7	Putative IQ motif and ankyrin repeat domain-containing protein LOC642574
Q495C1-3;Q495C1;Q495C1-2;Q495C1-4;Q495C1-5;Q495C1-6	Isoform 3 of Probable E3 SUMO-protein ligase RNF212
Q96AK3	DNA dC->dU-editing enzyme APOBEC-3D
Q15599;Q15599-2	Na(+)/H(+) exchange regulatory cofactor NHE-RF2
P63096;P63096-2	Guanine nucleotide-binding protein G(i) subunit alpha-1
Q02080-2	Isoform 2 of Myocyte-specific enhancer factor 2B
Q16527	Cysteine and glycine-rich protein 2
Q5VIY5;P17035;Q6ZN06;Q96IR2	Zinc finger protein 468
Q8N7H5;Q8N7H5-2	RNA polymerase II-associated factor 1 homolog
Q15884-2	Isoform 2 of Protein FAM189A2
Q10589-2;Q10589	Isoform 2 of Bone marrow stromal antigen 2
Q2T9K0;Q2T9K0-2;Q2T9K0-6;Q2T9K0-7	Transmembrane protein 44
Q9BWW8	Apolipoprotein L6
Q9NW61	Pleckstrin homology domain-containing family J member 1

Q9H503;Q9H503-2	Barrier-to-autointegration factor-like protein
O60264	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5
P07197	Neurofilament medium polypeptide
P62079	Tetraspanin-5
Q9NPB3-2	Isoform S-CaBP2 of Calcium-binding protein 2
P49863	Granzyme K
O95372	Acyl-protein thioesterase 2
Q3SYG4-2;Q3SYG4;Q3SYG4-3;Q3SYG4-4	Isoform 2 of Protein PTHB1
P07902	Galactose-1-phosphate uridylyltransferase
Q9H4G1	Cystatin-9-like
Q6IEG0-2	Isoform 2 of U11/U12 small nuclear ribonucleoprotein 48 kDa protein
P02774-2	Isoform 2 of Vitamin D-binding protein
Q9H6B4	CXADR-like membrane protein
Q13609-2;Q13609	Isoform 2 of Deoxyribonuclease gamma
Q15526-2	Isoform 2 of Surfeit locus protein 1
P01042-3	Isoform 3 of Kininogen-1
P41208	Centrin-2
Q16890	Tumor protein D53
Q86T03-3	Isoform 3 of Type 1 phosphatidylinositol 4,5-bisphosphate 4-phosphatase
Q495M9	Usher syndrome type-1G protein
Q13287	N-myc-interactor
P14854	Cytochrome c oxidase subunit 6B1
Q8WU39;Q8WU39-3	Marginal zone B- and B1-cell-specific protein
Q5JXB2	Putative ubiquitin-conjugating enzyme E2 N-like
Q96EV2	RNA-binding protein 33
Q86YP4-2;Q86YP4;Q86YP4-3	Isoform 2 of Transcriptional repressor p66-alpha
P52788;P52788-2	Spermine synthase
Q9BZW8-2;Q9BZW8;Q9BZW8-4	Isoform 2 of Natural killer cell receptor 2B4
P01782	Ig heavy chain V-III region DOB
Q8IY31-2	Isoform 2 of Intraflagellar transport protein 20 homolog
Q5VWG9	Transcription initiation factor TFIID subunit 3
Q8WU43	Uncharacterized protein C2orf15
Q9Y3D0	Mitotic spindle-associated MMXD complex subunit MIP18
P31629	Transcription factor HIVEP2
O60927	Protein phosphatase 1 regulatory subunit 11
Q3B8N5;Q3B8N5-2	Prospero homeobox protein 2
P00739	Haptoglobin-related protein
Q96D21	GTP-binding protein Rhes
P09132	Signal recognition particle 19 kDa protein
Q8WXX5	DnaJ homolog subfamily C member 9
Q9HAS0	Protein Njmu-R1
Q9H467	CUE domain-containing protein 2
P10092	Calcitonin gene-related peptide 2

Q5VST9-6	Isoform 5 of Obscurin
Q96P26;Q96P26-2;Q96P26-4	Cytosolic 5'-nucleotidase 1B
Q9C0K1-3;Q9C0K1	Isoform 3 of Zinc transporter ZIP8
Q8N8X6;Q8N8X6-2	Golgin subfamily A member 2-like protein 4
P21912	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial
Q8IXQ4	GPALPP motifs-containing protein 1
Q9NWX6	Probable tRNA(His) guanylyltransferase
P01623;P18135	Ig kappa chain V-III region WOL
P06889	Ig lambda chain V-IV region MOL
Q9H1F0	WAP four-disulfide core domain protein 10A
Q9H8M7-2	Isoform 2 of Protein FAM188A
Q9UHQ7	WW domain-binding protein 5
O15392-2;O15392	Isoform 2 of Baculoviral IAP repeat-containing protein 5
Q9H813	Transmembrane protein 206
Q8WUH6	UPF0444 transmembrane protein C12orf23
Q8IYX7	Protein FAM154A
Q96EE3-1	Isoform B of Nucleoporin SEH1
Q58EX7-3	Isoform 3 of Puratrophin-1
Q9BYJ0	Fibroblast growth factor-binding protein 2
O43768-7;O43768-3;O43768-5;O43768-6;O43768-9	Isoform 7 of Alpha-endosulfine
Q96EE3	Nucleoporin SEH1
Q9NRW1	Ras-related protein Rab-6B
Q8N8H1	Putative protein ZNF321
Q9BQI0-4	Isoform 4 of Allograft inflammatory factor 1-like
Q9Y2M5	Kelch-like protein 20
Q502X0	MORN repeat-containing protein 2
Q8IUC8	Polypeptide N-acetylgalactosaminyltransferase 13
Q8WV99	AN1-type zinc finger protein 2B
Q30201-2;Q30201;Q30201-10;Q30201-3;Q30201-4;Q30201-5;Q30201-6;Q30201-7	Isoform 2 of Hereditary hemochromatosis protein
Q9NQ30-2	Isoform 2 of Endothelial cell-specific molecule 1
Q6ZN54-2	Isoform 2 of Differentially expressed in FDCP 8 homolog
Q15773	Myeloid leukemia factor 2
O60431	Olfactory receptor 1I1
Q96GE6;Q96GE6-2;Q96GE6-3;Q96GE6-4	Calmodulin-like protein 4
Q86Y33;Q86Y33-2;Q86Y33-3	Cell division cycle protein 20 homolog B
Q9BXF9	Tektin-3
Q9C0D0;Q9C0D0-2	Phosphatase and actin regulator 1
Q29983	MHC class I polypeptide-related sequence A
P59796	Glutathione peroxidase 6
Q2TAY7	WD40 repeat-containing protein SMU1
Q496A3	Spermatogenesis-associated serine-rich protein 1
Q12912;Q12912-2	Lymphoid-restricted membrane protein
O14653-3;O14653;O14653-2	Isoform 3 of Golgi SNAP receptor complex member 2

Q14CN4-2;Q14CN4;Q14CN4-3	Isoform 2 of Keratin, type II cytoskeletal 72
Q8TF39;Q8TF39-2	Zinc finger protein 483
Q9BVN2;Q9BVN2-2	RUN and SH3 domain-containing protein 1
Q16831	Uridine phosphorylase 1
Q7Z572	Spermatogenesis-associated protein 21
Q14247;Q14247-3	Src substrate cortactin
Q08174-2;Q08174;Q08174-3	Isoform 2 of Protocadherin-1
Q3KNT9;Q3KNT9-2;Q3KNT9-3	Transmembrane protein 95
Q14244-7;Q14244;Q14244-2;Q14244-3;Q14244-4;Q14244-5;Q14244-6	Isoform 7 of Ensconsin
Q9Y6V0-5;Q9Y6V0-6	Isoform 5 of Protein piccolo
O60312	Probable phospholipid-transporting ATPase VA
P10909-3	Isoform 3 of Clusterin
Q9Y2T2	AP-3 complex subunit mu-1
P23510	Tumor necrosis factor ligand superfamily member 4
Q9H2F9	Coiled-coil domain-containing protein 68
Q6A163	Keratin, type I cytoskeletal 39
P16152	Carbonyl reductase [NADPH] 1
O95057	GTP-binding protein Di-Ras1
P52945	Pancreas/duodenum homeobox protein 1
P19397	Leukocyte surface antigen CD53
P55082	Microfibril-associated glycoprotein 3
O75818-2;O75818	Isoform 2 of Ribonuclease P protein subunit p40
Q15149-6	Isoform 6 of Plectin
Q9UF47	DnaJ homolog subfamily C member 5B
Q7Z4N8-2;Q7Z4N8	Isoform 2 of Prolyl 4-hydroxylase subunit alpha-3
P21695;P21695-2	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic
Q5R372-5;Q5R372-6;Q5R372-7;Q5R372-8	Isoform 5 of Rab GTPase-activating protein 1-like
Q15149;Q15149-2;Q15149-3;Q15149-4;Q15149-5;Q15149-7;Q15149-9;Q6FI81;Q6FI81-3	Plectin
O60260-2;O60260	Isoform 2 of E3 ubiquitin-protein ligase parkin
P04424;P04424-2;P04424-3	Argininosuccinate lyase
Q05BQ5;Q05BQ5-3	MBT domain-containing protein 1
P35237	Serpin B6
P13727	Bone marrow proteoglycan
P48775	Tryptophan 2,3-dioxygenase
Q13325	Interferon-induced protein with tetratricopeptide repeats 5
P69891;P69892	Hemoglobin subunit gamma-1
A1A5D9	Bicaudal D-related protein 2
Q9Y2V0	Uncharacterized protein C15orf41
Q3B7I2	Protein canopy homolog 1
Q96RU2;Q96RU2-2	Ubiquitin carboxyl-terminal hydrolase 28
Q14691	DNA replication complex GINS protein PSF1
P22460;P22460-2	Potassium voltage-gated channel subfamily A member 5
P0DJJ0	SLIT-ROBO Rho GTPase-activating protein 2C

Q8IY22-3;Q8IY22;Q8IY22-2	Isoform 3 of C-Maf-inducing protein
Q9BVQ7;Q9BVQ7-2;Q9BVQ7-3	Spermatogenesis-associated protein 5-like protein 1
Q6P6B1-2;Q6P6B1	Isoform 2 of Uncharacterized protein C8orf47
P09884	DNA polymerase alpha catalytic subunit
Q8N4V1	Membrane magnesium transporter 1
Q96S95	Calcium/calmodulin-dependent protein kinase II inhibitor 2
Q8IVL5	Prolyl 3-hydroxylase 2
Q96CX2	BTB/POZ domain-containing protein KCTD12
A4D2P6	Delphilin
Q9NZ72	Stathmin-3
Q9NQW1-3	Isoform 3 of Protein transport protein Sec31B
Q5TC04	Putative uncharacterized protein ATP1A1OS
Q15149-8	Isoform 8 of Plectin
Q5VUM1	UPF0369 protein C6orf57
O75648-2;O75648	Isoform 2 of Mitochondrial tRNA-specific 2-thiouridylase 1

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