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Investigation into the effects of end stage renal failure and renal replacement therapy on adipose tissue metabolism

A DISSERTATION SUBMITTED TO THE INSTITUTE OF AGING AND CHRONIC DISEASE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CLINICAL CHEMISTRY

By

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ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ADAM8	Disintgrin and metallopeptidase domain 8
ANOVA	Analysis of variance
ASP	Acylation-stimulation protein
AT	Adipose tissue
ATGL	Adipose triglyceride lipase
AURORA	A Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Hemodialysis: An Assessment of Survival and Cardiovascular Events
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
C3	Complement factor 3
C/EBPα	CCAAT/enhancer binding protein-a
cAMP	Cyclic adenosine monophosphate
CAD	Coronary heart disease
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2
cDNA	Complementary DNA
CETP	Cholesteryl ester transfer protein
CKD	Chronic kidney disease
CRF	Chronic renal failure
CRP	C-reactive protein
CM	Chylomicron
CVD	Cardiovascular disease
C_T	Threshold cycle
4D	Die Deutsch Diabetes Dialysis
Da	Dalton
DAB	3, 3'-Diaminobenzidine
DG	Diacylglycerol

DGAT	Diglyceride acyltransferase
DMED	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
EFG	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESRD	End stage renal disease
FAS	Fatty acid synthase
FABP4	Fatty acid-binding protein 4
FCS	Foetal calf serum
FFA	Free fatty acid
G-3-P	Glyceraldehyde 3-phosphate
GFR	Glomerular filtration rate
GLP-2	Glucagon-like peptide-2
GLUT	Glucose transporter
HD	Haemodialysis
HDF	Haemodiafiltration
HDL	High-density lipoprotein
HMG CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMW	High molecular weight
HRP	Horseradish peroxidase
HSL	Hormone sensitive lipase
Hs-CRP	High-sensitivity CRP
IBMX	3-isobutyl-I-methylxanthine
IDL	Intermediate density lipoprotein
Ig	Immunoglobulin
IL	Interleukin
KCL	Potassium chloride
LCAT	Lecithin-cholesterol acyltransferase
LDH	Lactate dehydrogenase

LDL	Low density lipoprotein
LDL-R	Low density lipoprotein receptor
Lp (a)	Lipoprotein (a)
LPL	Lipoprotein lipase
LPR	Lipoprotein receptor-related protein
LR	Leptin recptor
MAT	Macrophage aidpose tissue
MCP-1	Monocyte chemoattractant protein-1
MDRD	Modification of Diet in Renal Disease
2-MG	2-Monoglyceride
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
MTP	Microsmoal triglyceride transfer protein
NADH	Nicotinamide adenine dinucleotide
Ob-R	Leptin receptor
PD	Peritoneal dialysis
Peri A	Perilipin A
PBS	Phosphate buffered saline
POLR2A	DNA-dependent RNA polymerase
PCR	Polymerase Chain Reaction
PKA	Protein kinase A
PNPAL2	Patatin-like phospholipase domain-containing 2
PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated fatty acid
RLUHT	the Royal Liverpool University Hospital Trust
RNA	Ribonucleic
acid RNase	Ribonuclease
RPM	Revolutions per minute
RT	Reverse transcription
S.E.M	Standard error of the mean
SD	Standard deviation
SGBS	Simpson-Golabi-Behmel syndrome

SREBP-1c	Sterol regulatory element binding protein-1c
T3	Triiodothyronine
TG	Triglycerides
TBE	Tris-borate-EDTA
Tm	Melting temperature
TNF-α	Tumour necrosis factor-α
Tris-HCl	Tris (hydroxymethyl) aminomethane Hydrochloride
TZD	Thiazolidinedione
UCP-1	Uncoupling protein 1
VLDL	Very low-density lipoprotein
WAT	White adipose tissue

DECLARATION

I declare that the content of this thesis entitled 'Investigation into the effects of end stage renal failure and renal replacement therapy on adipose tissue metabolism' is my own work carried out at both the Clinical Chemistry Department and the Obesity Biology Unit at the University of Liverpool.

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ABSTRACT

Background and aims: End stage renal disease (ESRD), an increasing global major public health problem, has greatly increased cardiovascular disease risk (CVD) than the general population for unknown reasons. Preliminary data suggest that adipose tissue, shown to contribute to CVD risk in non-ESRD populations, may also play a part in ESRD. Adipose tissue is a complex organ which not only functions as a fat storing depot, but also plays an essential role in lipid metabolism. Recently, researchers have recognized that adipose tissue is also an endocrine organ since it secretes several cytokines and adipokines including leptin, adiponectin and interlukin-6 (IL-6). Alteration in circulating adipokines has been shown in ESRD patients. The objective of the study was to clarify the role that adipose tissue and lipoproteins may have in ESRD. The work presented in this thesis has focused on the role of adipose tissue in ESRD patients undergoing haemodialysis and peritoneal dialysis. Both the nutrient and endocrine functions of adipose tissue were addressed in this thesis.

Materials and methods: Most studies were carried out on novel in vitro adipocyte assay system developed for these experiments. Novel approaches to investigate ESRD included isolating lipoprotein fractions (VLDL, LDL and HDL) by ultracentrifugation from ESRD and matched human controls followed by incubation with the normal adipocytes in the novel assay system developed for these experiments. One experiment involved collection of human adipose tissue samples. Given the scope of the thesis mainly gene expression data was collected on candidate genes analysed by quantitative real-time PCR in these novel pilot studies.

Results: An atherogenic lipoprotein profile was seen in ESRD patients. Haemodialysis patients had increased serum triglycerides and decreased HDL-cholesterol compared to the control subjects. Both haemodialysis and peritoneal dialysis groups had increased VLDL. The lipoprotein pattern in the present study also indicated the peritoneal dialysis group had more atherogenic lipoproteins, IDL and small dense LDL.

Expression of lipoprotein lipase, hormone-sensitive lipase and regulatory proteins of lipid metabolism complement protein 3 and low density lipoprotein receptor were downregulated when treated with uraemic lipoproteins *in vitro*. Serum concentrations of total adiponectin, high molecular weight adiponectin and leptin were significantly higher in the ESRD. Gene expression of adiponectin, IL-6 and leptin was lower in ESRD patients. The results showed a tendency towards an increased accumulation of macrophages in adipose tissue in ESRD patients.

Discussion: These findings are strongly suggestive of a defective storage function as well as impaired ability to release fatty acids consistent with dysfunctional adipose tissue in ESRD. These data strongly suggest that normal adipocyte function is altered when exposed to a uraemic environment *in vitro*. Macrophage infiltration into adipose tissue has been suggested to play role in insulin resistance and systemic inflammation. The results from the modulation of this *in vitro* adipocyte assay system not only serves as a convenient and unique model to study mechanisms of disease such as ESRD and its treatments but has so far also provided an initial insight that strongly suggests several mechanisms that could contribute to the CVD risk associated with ESRD.

Conclusion: An *in vitro* system to study the effect of uraemic lipoproteins and serum on adipose tissue nutrient and endocrine function through adipocyte gene expression was conducted and compared where available with the corresponding circulating protein measurements. **Further studies** in ESRD, both by broadening the number of adipocyte genes (and proteins) and undertaking a mechanistic approach to clarifying the reasons for the changes observed would be necessary and informative.

CHAPTER 1

INTRODUCTION

1.1 The Kidneys

The kidneys are two bean-shaped organs located outside the peritoneal cavity and close to the posterior part of abdomen wall. The kidney is complex organ and composed of nephrons which are the functional units in the kidney. There are approximately one million nephrons in each kidney. The kidneys are surrounded by a layer of fat and are loosely connected to the abdominal wall by connective tissue. The major function of the kidney is the excretion of metabolic waste from the body besides others including regulation of water and electrolyte balance, regulation of arterial blood pressure and regulation of vitamin D production (Eaton et al., 2009, Greenberg and Cheung, 2009).

Glomerular filtration rate (GFR) is a measure of kidney function. GFR is defined as the volume of plasma ultrafiltrate presented to the nephrons per unit time in the process of urine formation and expressed as millilitres per minute. Mean GFR values in young men and women are approximately 130 ml/min/1.73 m^2 and 120 ml/min/1.73 m^2 respectively (Schwartz and Furth, 2007). Traditionally, GFR estimation relied on timed urine collections and blood sampling and is associated with a number of pitfalls including incomplete collection. GFR measurements assume that the substance used to characterise GFR is freely filtered at the glomerulus and following filtration is neither reabsorbed nor secreted, thereby only reflecting the filtration function of the nephron. This is true in the case of solutes such as inulin but not others such as creatinine. Creatinine is not only filtered but also secreted by tubules thereby overestimating the GFR. In addition to inulin and creatinine, other approaches have employed substances such as 99mTcDTPA and 51CrEDTA. Although there are a large number of functions

performed by the kidney, traditionally GFR has been chosen as the measure that reflects function. Creatinine clearance has been the main approach to characterise GFR and is relatively easy to carry out compared to other measurements described earlier (Stevens et al., 2006b). The level of creatinine in the blood is a useful guide to kidney function since decreasing GFR leads to increasing creatinine. Therefore steady state serum creatinine can be used to estimate GFR (eGFR) using age, sex, and race, and this is often calculated by computer and reported along with the creatinine blood test. It is only an estimate. A number of equations have been developed to estimate the GFR based on creatinine measurements and is shown in Table 1.1 and in the UK some form of the MDRD equation is in use. eGFR is most likely to be inaccurate in people at extremes of body type, for example the malnourished and amputees. It is not valid in pregnant women or in children. The MDRD equation tends to underestimate normal or near-normal function, so slightly low values should not be over-interpreted. Some racial groups may not fit the MDRD equation well and this usually involves a correction for race. Furthermore, laboratory differences in creatinine estimations may lead to significant differences. The K/DOQI distinguishes 5 grades of severity of CKD and is shown in Table 1.2.

1.2 End-stage renal disease (ESRD)

End-stage renal disease (ESRD) is rapidly becoming one of the major public health problems worldwide. The third National Health and Nutrition Examination Survey (NHANESIII) reported that 11% of US adult population had diagnosed chronic kidney disease (CKD) (Kwan and Beddhu, 2007). In addition, it is estimated that the incidence of kidney disease has also increased (Mitka, 2008).

ESRD has many adverse outcomes which need to be monitored and managed. A wide variety of diseases including cardiovascular disease (CVD) and obesity are associated with ESRD.

CKD is defined as persistence for 3 or more months of either kidney damage or GFR less than 60 ml/min/1.73 m^2. Kidney damage can be detected by urinary testing for the presence of microalbuminuria, macroalbuminuria, abnormal urinary sediment or scanning the kidney by imaging tools (Fuster, 2008).

As already described in detail, GFR is considered the most useful measurement for assessing kidney function in clinical practice (Schwartz and Furth, 2007). The National Kidney Foundation in the USA support the use of a number of different predictive equations based on serum creatinine measurement to estimate GFR (Table 1.1) (2002, Stevens et al., 2006a). One method to estimated GFR, also employed in the current thesis, is the Modification of Diet in Renal Disease (MDRD) equation:

GFR (ml/min/1.73m^2) = 175x [serum creatinine (μmol/L)x0.011312$^{-1.154}$]x[Age]$^{-0.203}$x[1.212 if black] x [0.742 if female].

Renal Disease can be classified into stages according to the GFR and presence of structural damage (2002). Patients with stage I have normal GFR but can be recognized by either albuminuria or structural kidney damage. Patients with stage 5, (GFR rate < 15 ml/min/1.73 m^2) or End-stage Renal Disease (ESRD) require renal replacement therapy (2002) (Table 1.2).

Cockcroft-Gault formula

Estimated creatinine clearance (Cl_{cr}) = ((140-age (years))*weight (Kg^{10})*1.2/serum Cr (μmol/L))* 0.85 if female

Mayo Quadratic formula

Cl_{cr} = exp [1.911+5.249/SCr−2.114/SCr (μmol/L) 2−0.00686×age (years) −0.205 if female]

Schwartz formula

Cl_{cr} = (k*Height)/ serum Cr

Table1.1 Commonly used formulae for estimating kidney function

ESRD is the final common pathway for all renal disease and causes include factors such as ischaemia, hypoxia, proteinuria and hypertension besides many others (Yu, 2003). In addition, there are various chemical mediators and several predisposing factors such as smoking, diabetes and hyperlipidaemia (Ejerblad et al., 2006, Stenvinkel et al., 2008, Yu, 2003).

Obesity is also one of the major risk factors for ESRD (Beddhu and Kwan, 2007, Wang et al., 2008b). Obesity mainly contributes to the progress of ESRD because of its role in pathogenesis of hypertension and Type 2 diabetes (Hall et al., 2004), but may also independently increase CKD risk. The focus of this thesis was on patients with ESRD.

1.3 Renal Replacement therapy

Replacement therapy, which includes dialysis and kidney transplantation, is required in end-stage disease. Transplantation is considered the best replacement therapy but due to the limited availability of organ donors dialysis is by necessity the alternative choice (Levey et al., 2003, Levey et al., 2005).

Dialysis may involve haemodialysis (HD) or peritoneal dialysis (PD). There are various methods of HD including haemofiltration (HF), haemodiafiltration (HDF) and acetate-free biofiltration (AFB) (Kannaiyan et al., 2006). Haemodialysis works on the basis of diffusion of substances from blood into another suitable fluid when it is separated by a semi-permeable membrane. Molecules are cleared from blood depending on their molecular weight and electrical charge. Molecules that have molecular weight greater than 60,000 Da cannot be cleared while those less than 60 Da can be easily cleared (Murugan and Ken, 2007). High flux dialysis

membranes have been recently used to improve the clearance of middle-range molecules (between 500 to 30,000 Da) (Himmelfarb and Ikizler, 2010, Murugan and Ken, 2007).

Patients can carry out peritoneal dialysis, in contrast to haemodialysis, at home rather than in hospital. Peritoneal dialysis is a technique where dialysis fluid is introduced into the peritoneal cavity. Molecules from the blood can be cleared depending on their concentration gradients (Tim and Martin, 2007). Although PD is more convenient than haemodialysis, it has drawbacks which include chronic inflammation (Lai and Leung, 2010). Peritoneal dialysis is often used in someone just starting dialysis (Oreopoulos et al., 2009).

Patients crucially require frequent dialysis to prevent or treat short-term fatal metabolic abnormalities like hyperkalaemia and acidosis in addition to treating other complications of chronic kidney disease (Ifudu, 1998). However, the longer term efficacy of dialysis is limited since morbidity and mortality remains high. High morbidity due to cardiovascular, nutritional, haematological, sexual, gastrointestinal and neurological problems persist (Hill, 2007, Ifudu, 1998).

A number of studies reported that mortality in patients who are undergoing dialysis is around 40-60 percent (Miller et al., 2010) (Figure 1.1). This increased mortality clearly depends on the age of patients, duration if disease as well as many other factors including the underlying cause of the renal failure. The kidney is central to excretion of potentially toxic waste products in urine. In ESRD the inability to excrete these products effectively causes their accumulation; these have been termed uraemic toxins, which have profound biological effects having an adverse impact on organs of the body (Vanholder et al., 2008a).

It has been suggested that many factors make dialysis less efficient as treatment compared with renal transplantation or normally functioning kidneys. These factors include the variability in the clearance of small molecules, inability to clear protein-bound molecules and the technical factors from the dialysis process itself like dialysate flow (Raff et al., 2008).

1.4 Cardiovascular risk factors in ESRD

It is well established that patients with ESRD have a high incidence of CVD compared to the general population. Moreover, CVD is considered the major cause of morbidity in ESRD patients (Sarnak et al., 2003). This association has not been fully understood and cannot be adequately explained by changes in the traditional risk factors (Stenvinkel, 2010).

Since CVD is a predominant feature in ESRD, early diagnosis of kidney impairment as a prelude to better preventative management could reduce CVD complications. In addition to early diagnosis, a fuller understanding of the causative factors is necessary in order to apply appropriate interventions that can decrease CVD prevalence in ESRD (Anavekar and Pfeffer, 2004, Yamamoto and Kon, 2009) .

Non-traditional risk factors may contribute to the development of CVD in ESRD patients as many studies have suggested (Rucker and Tonelli, 2009, Sarnak, 2003, Sarnak and Levey, 2000, Zoccali et al., 2004).

Stage	GFR	Description
1	≥ 90	Normal or increased GFR, with other evidence of kidney damage
2	60–89	Slight decrease in GFR, with other evidence of kidney damage
3A	45–59	Moderate decrease in GFR, with or without other evidence of kidney damage
3B	30–44	
4	15–29	Marked decrease in GFR, with or without other evidence of kidney damage
5	< 15	Kidney failure

Table 1.2 Stages of chronic kidney disease

GFR, Glomerular filtration rate
CKD staging system is mainly based on measured or estimated GFR.

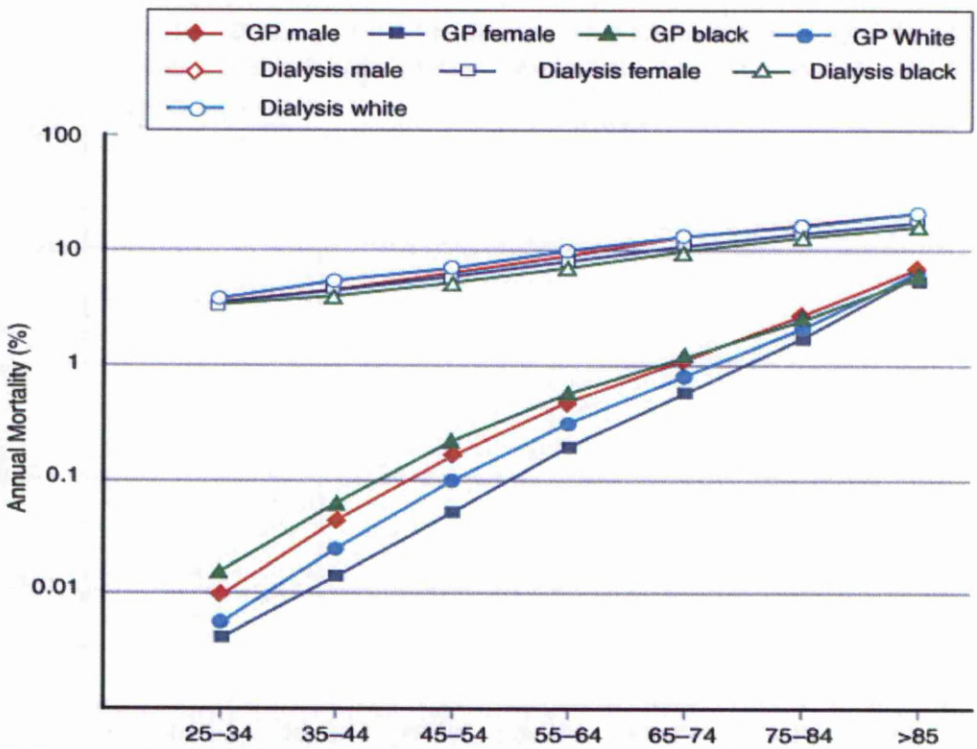

Figure 1.1: Cardiovascular mortality in the general population and in ESRD treated by dialysis

CVD mortality includes death due to arrhythmias, cardiomyopathy, cardiac arrest myocardial infarction, atherosclerotic heart disease and pulmonary edema. ESRD is compared with the general population.

Risk factors such as hypercholesterolaemia, very important in the general population, appear to have less impact in terms of CVD mortality in ESRD (Yamamoto and Kon, 2009). One of the leading causes of ESRD is diabetes mellitus, and the attendant dyslipidaemia is commonly seen.

Even in non-diabetic ESRD, however, a higher prevalence of hypertension, dyslipidaemia and uraemia-related risk factors have been shown (Collins et al., 2003, Tzanatos et al., 2009). Paradoxically, although statins decrease CVD in diabetes, they show less convincing benefit in improving the outcome of CVD in ESRD (Fellstrom et al., 2009). The focus of the studies in the present thesis is mainly non-diabetic ESRD.

1.5 Adipose tissue

Traditionally adipose tissue was considered to be a mainly a storage depot for dietary triglycerides. However, it is now also recognized to be an endocrine organ. This finding has attracted more research focusing on how adipokines may influence pathophysiological conditions.

Adipose tissue is highly specialized and not homogenous consisting of a variety of different cell types. These cells include adipocytes, preadipocytes, macrophage, endothelial cells, fibroblasts and leukocytes (Beddhu and Kwan, 2007) .

The adipocytes (and other cells in adipose tissue) produce cytokines, chemokines, growth and complement factors which may have a role in ESRD (Chudek and Wiecek, 2006, Trayhurn, 2007). A major function of adipocytes is to store energy in the form of triglycerides during the fed-state and release the stored energy in the form of fatty acids in the post-absorptive state (Frühbeck et al., 2001).

Adipose tissue may be classed histological and functionally as white (WAT) or brown (BAT). WAT can be found distributed in different compartments (depots), subcutaneous or visceral, each type displaying unique metabolic characteristics. Subcutaneous adipose tissue can expand outward and is mainly used for long-term energy storage. In contrast, visceral adipose tissue depot is smaller but is an important source of short-term energy (Lefebvre et al., 1998). The expansion of these depots may result due to enlargement of existing adipocytes (hypertrophy) and formation of new adipocytes (hyperplasia) (Karastergiou and Mohamed-Ali, 2010).

WAT is characterized by having more lipid content and free fatty acid release via lipolysis. In contrast, BAT is more cellular with highly packed mitochondria and highly vascularised. Many factors have been described to increase fat accumulation and include environmental and genetic factors (Bays et al., 2008). Subcutaneous adipose tissue is considered less metabolically active compared with visceral. Visceral adipose tissue has been known to be associated with metabolic risk factors, comprising the metabolic syndrome as well being as an important predictor of coronary artery disease (Shen et al., 2006). The present thesis is focused on WAT.

1.5.1 Brown adipose tissue (BAT)

BAT is present in significant amounts in humans during fetal life and the number decreases progressively with ageing. BAT cells are spherical and contain multilocular fat droplets with diameters ranging from 10 to 50 um. BAT, under the electron-microscope, is clearly distinguished by prominent peroxisome, Golgi complex, rough and smooth endoplasmic reticulum (Cinti, 2007). In addition,

numerous mitochondria are found since BAT has the main responsibility for thermogenesis. This activity is achieved by uncoupling protein 1 (UCP1), which are located in the inner mitochondria, and this protein is considered as a marker of brown adipocytes (Cinti, 2005).

1.5.2 White adipose tissue (WAT)

WAT represents about 15 to 25% of body weight in health reaching more than 50% in obesity (Mullerova and Kopecky, 2007). WAT is considered the predominant type in adult humans. Its functions include energy storage, insulation and protection of vital organs. The present studies focus on white adipose tissue function in culture *in vitro* as well as *in vivo*.

The nutrient function of the adipose tissue is important to allow optimal lipoprotein metabolism. The interaction of triglyceride-rich lipoproteins with adipose tissue is crucial in this regard and is an important focus of present studies. Various proteins and enzymes are involved in the lipoprotein-adipocyte interaction including lipoprotein lipase and hormone-sensitive lipase. Fat storage results from the uptake of fatty acids following the action of lipoprotein lipase on triglyceride-rich lipoproteins. There is limited de novo lipogenesis in adipose tissue (Kolditz and Langin, 2010).

1.6 Development of adipocytes

In view of the foregoing account highlighting the importance of adipose tissue function, it is necessary to analyse and understand adipose tissue development to better understand its role in health and disease. Adipogenesis takes place in both

the prenatal and postnatal states. However, the primary adipogenesis starts early in the prenatal period at the 14^{th} to 16^{th} week followed by secondary adipogenesis after the 23^{rd} week of gestation. Like all connective tissue cells, adipocytes originate from mesenchymally-derived stem cells (Ross, 2003). The process of adipogenesis includes preadipocyte proliferation and adipocyte differentiation (Gregoire et al., 1998).

After differentiation as shown in figure 1.2, the lipoblast develops from the small vessels in the fetus. When lipoblasts differentiate, smooth-surface vesicles begin to increase with decline in number of rough endoplasmic reticulum. Consequently, small lipid inclusions and pinocyotic vesicles appear. Then, mature adipocytes increase in size and become spherical and lipid mass compresses the nucleus to an acentric position (Ross, 2003) .

It is necessary for a cascade of specific cell signaling pathways, both stimulatory and inhibitory to be activated for adipogenesis to proceed. Peroxisome proliferator-activated receptor γ (PPARγ) activation is considered a key step, as it is required to induce adipocyte differentiation in fibroblasts and to maintain the differentiated state (Rosen and MacDougald, 2006). PPARγ activates many important adipose tissue specific genes including fatty acid binding proteins, lipoprotein lipase, acyl coenzyme A synthase and phosphoenolpyruvate carboxykinase (Otto and Lane, 2005). Along with PPARγ, many CCAAT-enhancing binding proteins (C/EBPs) are also required for adipogenesis (Farmer, 2010).

It has been shown that different depots of WAT have different appearances and functions during development suggesting a possible difference of their origin

(Billon and Dani, 2011). The gene expression profiles of cultured primary preadipocytes extracted from different depots of human WAT were analysed in one study and around 503 out of more than 900 genes identified differ among depots suggesting a heterogeneous developmental origin between different WAT depots (Tchkonia et al., 2007). This has provided more understanding of the adipose tissue development.

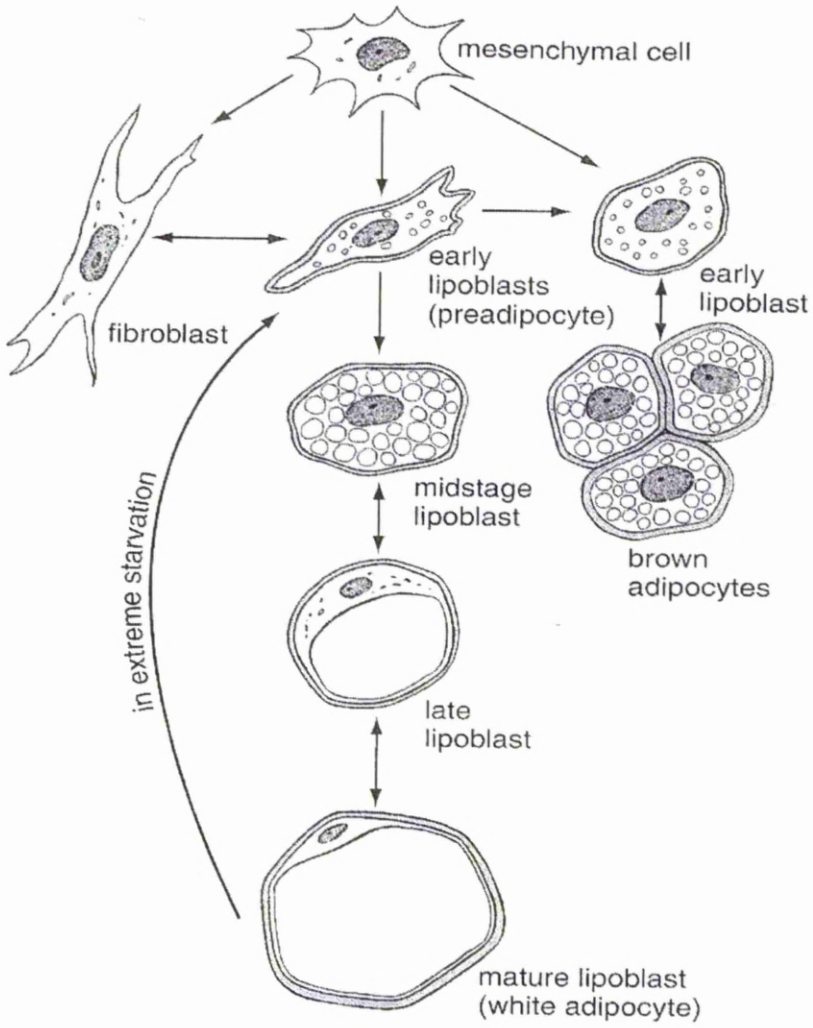

Figure1.2: Development of adipose tissue.

Adipocytes are derived from mesenchymal cells. One of the key adipogenesis steps is the characteristic alteration in cell shape as the cells convert from fibroblast (preadipocyte) to spherical morphology (adipocyte). Several key regulators involved in the adipogenesis include C/EBPα and PPAR γ.

Adapted from :(Ross, 2003)

1.7 Study of adipose tissue function using *in-vitro* and *in-vivo* models

White adipose tissue has been studied by several methodologies in order to investigate its function and structure. The use of experimental models is a crucial and basic step of experimental biology. *In vitro* and *in vivo* systems have been used for study of adipose tissue and have their individual advantages and disadvantages (Trayhurn and Beattie, 2001).

White adipose tissue is a heterogeneous organ that is composed of many cells types including adipocytes, macrophages, preadipocytes, endothelial and epithelial cells (Karastergiou and Mohamed-Ali, 2010). Many methods have been used to isolate the different adipose tissue fractions (Poulos et al., 2010).

I*n vivo* studies can better approximate physiological conditions prevailing in the human body whereas the *in vitro* studies employing cultured single cell types have the advantage of better control of the physiochemical environment in adipocytes (Freshney, 1994, Poulos et al., 2010). In terms of gene expression, determining whether the expression is a result of adipocytes or other cells is important (Trayhurn and Beattie, 2001). Changes in expression can be confirmed by analysing blood collected from the venous drainage. Alternatively, in situ hybridisation for the direct localization of mRNA can be used (Trayhurn and Beattie, 2001).

In vitro studies can be carried out by using cell lines or primary cell cultures. Human adipocytes, from different cell lines, show differences in metabolism and endocrine function. One such cell line, is the mouse 3T3-L1, which following clonal expansion is used for in vitro studies (Jones, 1996).

Primary adipocytes can be cultured by employing different methodologies including culturing of adipose tissue explants, culturing freshly isolated mature adipocytes or by culturing stromal cell fraction of adipose tissue (Jones, 1996). Moreover, knockout models can also be useful in studying specific factors in adipose biology (Poulos et al., 2010).

Human Simpson-Golabi-Behmel Syndrome (SGBS) preadipocyte cell strain has been used as a human primary adipocyte model. SGBS cells are characterized by stable adipogenic differentiation for many generations during their proliferation (Fischer-Posovszky et al., 2008). SGBS was developed from stromal-vascular fraction within adipose tissue (Wabitsch et al., 2001). Primary human preadipocytes are also available commercially from different sources like PromoCell and Zen-Bio. In this thesis, the SGBS and Promo Cell human adipocytes were employed as *in vitro* adipose tissue models.

1.8 Adipose tissue as endocrine gland

Adipose tissue was considered solely an energy storage organ for a long time. However, modern techniques and advances have lead to a better understanding of adipose tissue biology. The discovery of leptin secretion from adipocytes changed our perception of adipose tissue from a purely storage organ to one which also has a substantial endocrine activity. The endocrine function of the adipose tissue involves the production and secretion of a large number of hormones of which are the focus of the present study. The best known among these factors is leptin; an adipocyte-derived hormone that acts in the hypothalamus and decreases food intake, increases energy expenditure, and in peripheral tissue modulates

metabolism and other cellular functions. In addition to leptin, many other factors have been identified including tumour necrosis factor (TNF-α), adiponectin (also known as Acrp30 and AdipoQ), plasminogen activator–inhibitor, interleukin 6, resistin, transforming growth factor-β, adipsin and retinol-binding protein (Ahima and Osei, 2008, Trayhurn and Wood, 2004). The adipokines that are the focus of present studies are briefly reviewed.

1.8.1 Leptin

Leptin is an important adipocyte product being the first adipokine to be identified in 1994. It is a 16 KDa protein and encoded by ob gene (Zhang et al., 1994). Initially, leptin was thought as an obesity inhibitor in rodents. In humans it appears to be a hormone signal indicating energy deficiency (Maury and Brichard, 2010). Obesity is associated with high levels of plasma leptin which may reflect leptin resistance (Ahima and Osei, 2008). Leptin is produced mainly by adipocytes and also by other sites including skeletal muscle and placenta (Wolf et al., 2002) .

Leptin has many functions elicited through specific interaction with its receptor (obR) found in most tissues. Leptin has many actions including those in the brain, pancreas, liver and immune system. Mainly, leptin inhibits food intake by its primary action on the brain (Ahima, 2006, Trayhurn and Beattie, 2001, Galic et al., 2010). Its action on the brain and in particular the hypothalamus was linked subsequently to the energy homeostasis and reproductive function (Badman and Flier, 2007). Leptin receptor (ob-R) is found in the brain in high concentrations; in adipose tissue and skeletal muscle ob-R is found in low concentrations (Tartaglia, 1997).

Adipose tissue mass has been reported to correlate positively with plasma leptin levels (Wolf et al., 2002). Leptin levels are high in chronic renal failure (CRF) patients especially those undergoing PD (Heimburger and Stenvinkel, 2005) and its role in the nutritional status in ESRD has been studied (Kwan and Beddhu, 2007). Increased plasma levels of leptin have been proposed to contribute to malnutrition of uraemia as an indicator of low protein intake (Young et al., 1997).

Leptin has been shown to be excreted by the kidney (Cumin et al., 1996). Moreover, leptin has been proposed to play a role in inflammation (Kataoka and Sharma, 2006). Nordfors and colleagues showed that leptin gene expression in biopsy studies of adipose tissue is decreased in chronic renal failure (Nordfors et al., 1998) even though circulating concentrations are increased suggesting a major role for the kidneys in the degradation and elimination of leptin.

1.8.2 Adiponectin

Adiponectin is a 30 kDa protein that is produced only by mature adipocytes and is a member of soluble defence collagen family (Ahima, 2006, Bauche et al., 2006). Adiponectin structure comprises 244 amino acids and it belongs to the complement 1q family that contains a carboxyl terminal globular domain and amino terminal collagenous domain (Hu et al., 1996). Adiponectin expression increases during the preadipocyte differentiation to mature adipocytes (Körner et al., 2005). Human adiponectin has been observed in three different forms; low, middle and high molecular weights. The low molecular weight adiponectin is a tri-mer, middle molecular weight is a hexa-mer whereas the high molecular fraction is a dodeca-mer (Shen et al., 2008). Each adiponectin monomer has three domains; a variable N-terminal region, an α-helical collagenous and distinctive C-

terminal globular domains (Shapiro and Scherer, 1998). These forms are different in their biological action (Sowers, 2008). The high molecular weight is considered the active form (Schraw et al., 2008). High molecular weight form was also found to correlate with insulin resistance better than total adiponectin (Hara et al., 2006). The importance of high molecular weight adiponectin form has led to an assay being developed for the specific measurement of the high molecular weight fraction (Tanita et al., 2008).

Two receptors have been identified; AdipoR1 and 2. AdipoR1 and AdipoR2 are expressed in many tissues and organs but predominantly in muscle cells and liver cells, respectively (Beige et al., 2009, Shen et al., 2008). AdipoR1 has been showed to be involved in the inhibition of gluconeogenesis and increased fatty acid oxidation through the activation of AMPK pathways. AdipoR2 has also been showed to activate fatty acid oxidation in skeletal muscle and suppress oxidative stress and inflammation through the activation of PPAR-α pathway (Ouchi et al., 2000, Yamauchi et al., 2002, Yoon et al., 2006). Any dysregulation of these pathway might result in accumulation of triglycerides, inflammation and oxidative stress (Shen et al., 2008).

Unlike other adipokines, adiponectin has a negative correlation with the BMI (Brichard et al., 2003). Adiponectin has antiatherogenic, anti-inflammatory and insulin sensitizing functions (Bauche et al., 2006). Adiponectin inhibits oxidised LDL-mediated cell proliferation, lipid accumulation in monocyte-derived macrophages and transformation of macrophages into foam cells (Chudek and Wiecek, 2006).

In contrast to leptin, adiponectin is negatively associated with WAT mass (Ahima, 2006). Adiponectin shows a negative correlation between its plasma levels and BMI (Hu et al., 1996). Surprisingly, although low plasma adiponectin concentrations are associated with coronary artery disease and type 2 diabetes, plasma adiponectin is high in uraemia suggesting that the kidney may again be an important site for degradation and elimination (Chudek and Wiecek, 2006, Huang et al., 2004). This is rendered more likely in view of data suggesting that adiponectin gene is downregulated in ESRD (Marchlewska et al., 2004). Despite the paucity of data, what evidence there is suggests that circulating adipokine concentrations are altered in ESRD. Circulating levels of adiponectin are markedly elevated in ESRD (Wiecek et al., 2002). Adiponectin is also inversely associated with other metabolic risk factors, such as body mass index, blood glucose, insulinaemia as well as serum triglyceride and HDL cholesterol levels (Zoccali et al., 2003).

1.8.3 Interleukin-6

Interleukin-6 (IL-6) is a 22 to 27 kDa polypeptide produced by many cells including adipocytes, macrophage, fibroblast and endothelium (Pecoits-Filho et al., 2003). About 15-35% of detected plasma levels of interleukin-6 is produced by adipocytes (Mohamed-Ali et al., 1997).

It has been reported that plasma levels of interleukin-6 are positively correlated with adipose tissue mass (Bastard et al., 2007). Interleukin-6 is a proinflammatory mediator induced by stress and activates an acute phase protein response in the liver (Frühbeck et al., 2001). With regard to adipose tissue production, Interleukin-6 is produced mainly in visceral adipose tissue, a fat depot known to

be associated with insulin resistance and inflammation (Maury and Brichard, 2010) .

Interleukin-6 has been found to have a significant relationship with plasma high sensitivity C-reactive protein (hsCRP) in dialysis patients (Kwan and Beddhu, 2007). Interleukin-6 activates fatty acid oxidation and glucose uptake in skeletal muscle which might contribute to insulin resistance (Galic et al., 2010).

Interleukin-6 is involved in insulin resistance by inhibiting insulin signaling pathway through impairment of insulin-induced insulin receptor and IRS-1 phosphorylation in adipocytes and hepatocytes (Galic et al., 2010). Interleukin-6 plasma levels have been found to be increased in ESRD (Taskapan et al., 2007, Wang et al., 2008b).

1.8.4 Monocyte chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1) is a 13 KDa protein produced by the expression of the CCL2 gene on chromosome 17 (Yadav et al., 2010). Monocyte chemoattractant protein-1 is produced by variety of cell types including macrophage, endothelium and adipocyte (Kanda et al., 2006).

Monocyte chemoattractant protein-1 has been suggested to contribute to inflammation in adipose tissue (Kanda et al., 2006). Monocyte chemoattractant protein-1 binds to its receptor CCR2 in monocyte recruitment which is involved in many biological inflammation related processes (Yadav et al., 2010).

It has been reported that Monocyte chemoattractant protein-1 expression correlates with adipocyte size (Sartipy and Loskutoff, 2003), with one exception being the epicardial adipocytes (Eiras et al., 2010). It has been found that

proinflammatory markers like interleukin-6 can induce Monocyte chemoattractant protein-1 expression (Karastergiou and Mohamed-Ali, 2010). Monocyte chemoattractant protein-1 plasma levels have been found to be increased in ESRD suggesting a possible role for MCP-1 in inflammation in ESRD (Stinghen et al., 2009) . Monocyte chemoattractant protein-1 and its receptor have been proposed to contribute to ESRD progression by initiation and progression of tubule-interstitial damage and/or glomerular lesions (Viedt and Orth, 2002).

1.9 Macrophage infiltration into adipose tissue

Macrophage infiltration into adipose tissue has been shown to contribute to increased expression of pro-inflammatory adipokines in obesity (Weisberg et al., 2003, Xu et al., 2003). BMI in human has also been found to correlate positively with macrophage infiltration (Weisberg et al., 2003).

Macrophage infiltration can be detected by using a specific macrophage protein expression like cluster of differentiation 68 (CD68) and disintegrin and metallopeptidase domain 8 (ADAM 8) among others (Khazen et al., 2005). The mechanism(s) underlying macrophage infiltration in adipose tissue are still not fully known. It has been suggested that adipose tissue expansion leads to some process that in turn contributes to the initiation of macrophage recruitment. These including fatty acid flux, vascularisation, increased leptin secretion, hypoxia and adipocyte cell death (Surmi and Hasty, 2008). Increased accumulation of macrophages in adipose tissue may contribute to systemic inflammation in obesity. Pro-inflammatory markers such as TNF-α, IL-6 and resistin have been shown to be produced by macrophages (Bouloumie et al., 2005). Lean adipose

tissue, on the other hand, when infiltrated by macrophages, exhibit an increase in expression of anti-inflammatory cytokine genes such as interleukin 10 (IL-10) (Lumeng et al., 2007).

Macrophages are heterogeneous in function and several types have been described. One type of macrophage, M1, has features that are typically considered to reflect these cells such as pro-inflammatory capacity as well as the ability to cause insulin resistance. It has been found that the expansion of adipose tissue is associated with classically activated macrophages M1 (Lumeng et al., 2007). Alternatively activated macrophages (known as M2), on the other hand, are associated with normal adipocyte function and insulin sensitivity.

1.10 Adipose tissue in End-stage renal disease

White adipose tissue has been known to be involved in the regulation of many biological processes including body weight homeostasis, carbohydrate and lipid metabolism, immunity and others. Studying adiposity-related metabolism will improve the understanding of how adipose tissue contribute to cardiovascular disease and therefore potentially contribute to the development of appropriate treatment(s) (Chudek et al., 2006) .

Obesity increases the risk of ESRD (Wang et al., 2008b) and plays a role in development and progression of kidney disease itself (Beddhu and Kwan, 2007). Body mass index was identified as a predictor for elevated serum creatinine, ESRD and chronic renal disease (Ritz, 2008). In addition, in dialysis patients, BMI is associated with several adverse cardiovascular features such as insulin resistance, diabetes, anaemia, inflammation, coronary calcification and carotid

atherosclerosis (Rutkowski et al., 2006, Zoccali and Mallamaci, 2008). Body mass index also has been considered a risk factor for mortality in patients on haemodialysis and at the same time was used as an indicator of the nutritional status of ESRD patients (Iglesias and Diez, 2010).

Patients on peritoneal dialysis are likely to have more fat mass due to glucose-containing peritoneal dialysis solution being instilled into the peritoneal cavity; the result is an intra-abdominal fat accumulation (Fernstrom et al., 1998). As discussed already, excess visceral adipose tissue is considered a risk factor for cardiovascular disease.

A number of adipokines have been proposed to play an important patho-physiological role in patients with ESRD. These adipokines are of special interest in ESRD since they may have a role in inflammation especially interleukin-6 and tumour necrosis factor-α (Axelsson et al., 2006a, Zoccali et al., 2003).

Obesity, especially when associated with metabolic syndrome, is considered a risk factor for CVD. In addition, it has been shown that ESRD is associated with obesity. Obesity in ESRD patients is associated with many complications such as hypertension, dyslipidaemia, insulin resistance and kidney damage. Obesity is associated with an expansion of adipose tissue mass and consequently a potential increase of adipokine secretion. A number of adipokines including leptin, TNF-α, MCP-1, IL-6 and adiponectin are linked to chronic inflammation. The contribution of adipokines to ESRD is still not fully understood. There is limited evidence about the effect of leptin on the kidney pathophysiology. Leptin is associated positively with increased fat mass. Leptin has been shown to stimulate

proliferation of endothelial cells leading to hypertrophy of mesangial cells and in turn results in stimulation of vascular inflammation and oxidative stress. Previous studies showed that ESRD patients have a significant increase in circulating adipokines. A number of studies have tried to explain the effect of obesity on kidney disease, although the current data remains poorly understood. However, some researchers have hypothesized mechanisms to explain the effect of obesity including endocrine changes, low-grade inflammation, oxidative stress and endothelial dysfunction (Iglesias and Diez, 2010). An improved understanding of obesity related mechanisms influencing the CVD in ESRD may contribute to development of new prevention and treatment approaches. The role of some adipokines in the pathogenesis of cardiovascular and renal diseases is studied in this thesis (chapter 5).

It has been shown that obesity correlates with macrophage infiltration into adipose tissue (Weisberg et al., 2003). Furthermore, macrophage accumulation has been known to contribute mainly to the production of inflammation markers (Weisberg et al., 2003). It has been found that adipose tissue in ESRD patients also has increased macrophage numbers (Roubicek et al., 2009). Understanding the contribution of the adipose tissue to ESRD more fully is therefore important.

1.11 Uraemic toxins

In health, kidneys potentially remove toxic compounds from the blood and eliminate these in the urine. In ESRD, the loss of the renal capacity to eliminate these toxic substances causes their accumulation in the body. These potentially toxic compounds have been termed uraemic toxins and can be classed into three

groups namely (1) small water-soluble compounds with a molecular weight less than 500kd, (2) middle molecules with a MW of more than 500kd and (3) protein-bound substances (Dhondt et al., 2000) . These are shown in Tables 1.3 and 1.4. Even as far back as 2003, more than ninety toxins were identified with molecular weights between 60 Da (urea) to 32,000 Da (Interleukin 1β [IL-1β]) (Vanholder et al., 2003). Uraemic toxins affect every organ system in the body including the cardiovascular system. One publication listed some of these substances affecting the cardiovascular system, the focus of this thesis, and is shown in Table1.5.

Classification	Characteristics	Prototypes	Toxicity
Small water-soluble molecules	MW < 500 Da, easily removed by any dialysis strategy	Urea, creatinine	Not necessarily toxic
Middle molecules	MW > 500 Da, removed only through large-pored membranes	β_2-M, leptin	Large array of biological impacts
Protein-bound molecules	Any MW, difficult to remove with any dialysis strategy	Phenols, i ndoles	Large array of biological impacts

Table 1.3 Current classifications of uraemic retention solutes

Adapted from (Vanholder et al., 2008b)

Small water-soluble compounds	Protein-bound compounds	Middle molecules
1-Methyladenosine	2-Methoxyresorcinol	Adrenomedullin
1-Methylguanosine	3-Deoxyglucosone	Atrial natriuretic
1-Methylinosine	CMPF	β_2-Microglobulin
ADMA	Fructoselysine	β-Endorphin
α-keto-δ-Guanidinovaleriate	Glyoxal	β-Lipotropin
α-N-Acetylarginine	Hippuric acid	Cholecystokinin
Arabinitol	Homocysteine	Clara cell protein
Argininic acid	Hydroquinone	Complement factor D
Benzylalcohol	Indole-3-acetate	Cystatin C
β-Guanidinopropionate	Indoxyl sulfate	DIP I
Creatine	Kinurenine	δ-Sleep-inducing
Creatinine	Kinurenic acid	Endothelin
Cytidine	Melatonin	Hyalur
Dimethyl	Methylglyoxal	onic
glycine	N^{ε}-Carboxymethyllysine	acid
Erythritol	p-Cresol	Interle
Guanidine	Pentosidine	λ-Ig Light chain
Guanidinoacetate	Phenol	Leptin
Guanidinosuccinate	p-OHhippurate	Methionine-
Hypoxanthine	Putrescine	Neuropeptide Y
Malondial	Quin	Parathyroid
dehyde	olinic	hormone
Mannitol	acid	Retinol binding
Myoinositol		
N^2,N^2-Dimethylguanosine		
N^4-Acetylcytidine		
N^6-Methyladenosine		
N^6-Threonylcarbamoyladenosine		
Orotic acid		
Orotidine		
Oxalate		
Phenylacetylglutamine		
Pseudouridine		
SDMA		
Sorbitol		
Taurocyamine		
Threitol		
Thymine		
Uracil		
Urea		
Uric acid		
Uridine		
Xanthine		
Xanthosine		

Table 1.4 List of uraemic retention solutes

Adapted from (Vanholder et al., 2008b)

Uremic toxins	Leukocytes	Endothelial cells	Smooth muscle cells	Thrombocytes
AGE	X	X	X	
AOPP	X	X		
AGE-β-microglobulin	X			
Angiogenin-DIP I	X			
β2-microglobulin	x	x	x	
Complement factor D	x			
Cytokines	x	x	x	x
Homocysteine	x	x	X	
Leptin	X	x		x
Ig-light chain	X			
Guanidines	x			
Indoxyl sulfate		X		
Phenylacetic acid		x		

Table 1.5 Some uraemic toxins shown to have a potential cardiovascular impact

AGE advanced glycation end products; AOPP advanced oxidation protein products;
(Vanholder et al., 2008b)

1.12 Lipoproteins

Lipoproteins are a family of assembled proteins and lipid particles. Lipoproteins play an important role in transport and metabolism of dietary lipids. Abnormal lipoprotein metabolism is associated with an increase in the risk of cardiovascular disease (CVD). Depending upon density, compositional and functional properties, the lipoproteins are divided into several classes. These classes are chylomicrons secreted from absorptive enterocytes, very low density lipoprotein (VLDL) secreted by hepatocytes, low density lipoprotein (LDL) produced by metabolism of VLDL, and high density lipoprotein (HDL) produced by the intestine and the hepatocytes (Havel, 2001, Jain et al., 2007). The main function of lipoproteins is transport of lipids (cholesterol and triglycerides) between different tissues (Habenicht et al., 1994). Lipoproteins contain varying proportions of triglyceride (TG), free cholesterol, esterified cholesterol, and phospholipids, besides a large number of proteins (Figure 1.3).

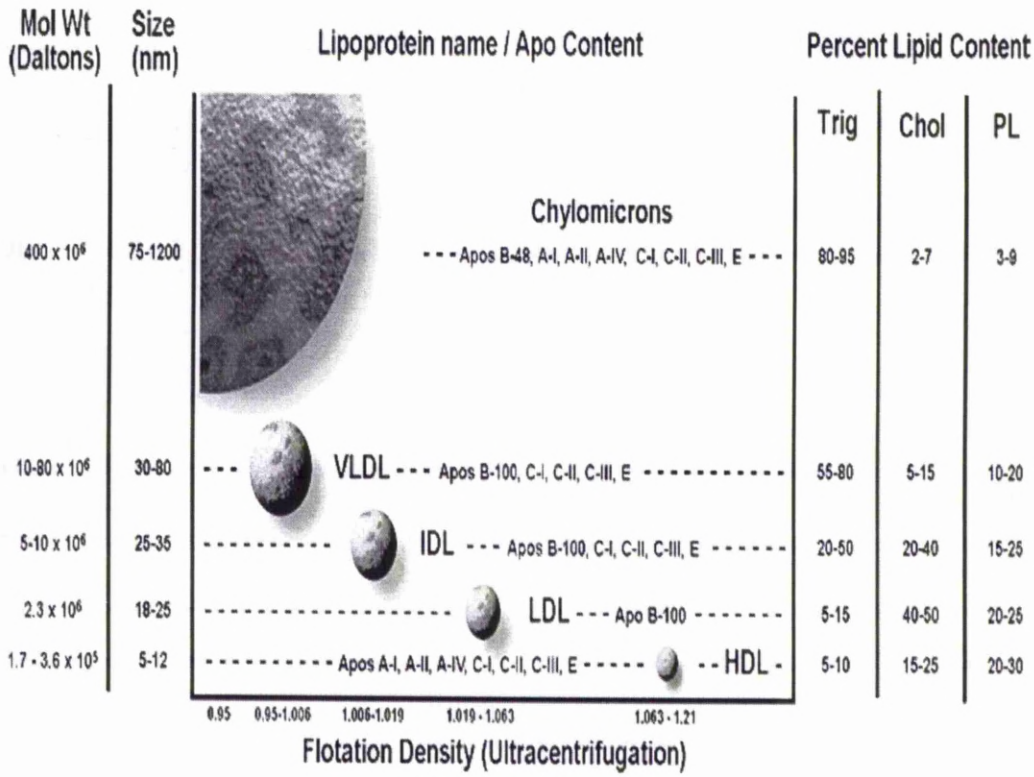

Figure 1.3: Characteristics of the Major Classes of Lipoproteins in Human Plasma

The most common classification of lipoproteins is based on flotation density/ultracentrifugation. The physical properties of the major lipoprotein classes are shown. Other common classification systems include differentiation by apolipoprotein content or electrophoresis.

Taken from (Saland and Ginsberg, 2007)

1.12.1 Chylomicrons (CM)

Chylomicrons are the largest lipoprotein particles with a molecular weight of 50-1000x10^6 Da. Chylomicrons are very rich in dietary TG and proportionally contain much less cholesterol, phospholipids and protein. Chylomicrons are produced in the intestines and have apo B-48 as the major protein as well as apo A-I, apo A-II and apo A-IV. Dietary TGs, diacylglycerol (DG), 2-monoglycerol (2-MG) and fatty acids are taken up by enterocytes in the small intestine to form nascent chylomicrons. Released nascent chylomicrons reach the venous circulation after traversing the thoracic duct. Interestingly, glucagon-like peptide 2 (GLP-2) increases chylomicron production in the intestine through the CD36 pathway (Kindel et al., 2010).

In the systemic circulation, nascent chylomicrons undergo several exchanges of apoproteins where apo C and apo E are transferred to nascent chylomicrons from HDL in exchange for apo A-I and apo A-IV (Vaziri, 2006). In the circulation, nascent chylomicrons bind to lipoprotein lipase (LPL) in endothelium of the capillaries in the skeletal muscle and adipose tissue. LPL mediated hydrolysis of TG leads to the liberation of fatty acids that can be taken up by those tissues for storage and metabolism (Figure 1.5). The residual particles, called remnant chylomicrons, is then released into the circulation and cleared by hepatocytes (Havel, 2001, Kindel et al., 2010, Vaziri, 2006). Apo C-II is required for LPL activation, apo E is required for binding of chylomicrons to the endothelium surface, whereas apo C III is an inhibitor of LPL (Redgrave, 2004). Following the hydrolysis of nascent chylomicron content, apo C and apo E are returned to HDL, and remnant chylomicrons are taken up by hepatocytes through LDL receptor-

related protein (LRP) (Redgrave, 2004, Vaziri, 2006). In addition to LRP, LDL receptor has been proposed to be involved in the clearance of remnant chylomicrons (Redgrave, 2004).

1.12.2 Very Low Density Lipoproteins (VLDL)

Next to chylomicrons, VLDL is also considered a large lipoprotein that contains TG as its main lipid content. VLDL molecular weight is 10-80x10^6 Da. The major structural proteins in VLDL are apo B-100 as well as apo E, apo A-I and apo A-II (Havel, 2001, Shelness and Sellers, 2001, Vaziri, 2006). Similar to chylomicrons, nascent VLDL also acquires apo C and apo E from HDL in the circulation. Together with chylomicrons, VLDL is considered as TG-rich lipoproteins. TG in VLDL is derived from de novo synthesis in hepatocytes or from plasma uptake of remnants and free fatty acids. These triglycerides are transferred into apo B-100 containing VLDL via the microsomal triacylglycerol transfer protein (MTP) (Shelness and Sellers, 2001). Following the secretion into the circulation, VLDL undergoes LPL-mediated hydrolysis similar to chylomicrons and this will lead to the formation of remnant VLDL which is also known as intermediate density lipoprotein (IDL) (Figure 1.6). One possible fate of IDL is uptake by the liver by LRP.

Unlike chylomicrons which are almost fully hydrolysed by LPL, LPL-mediated VLDL hydrolysis is partial and this can be explained by difference in the size. VLDL remnant particles are removed by VLDL receptor in myocytes and hepatocytes (Takahashi et al., 2004). Alternatively, VLDL undergoes further lipolysis by hepatic triacylglycerol lipase action leading to the formation of cholesterol-rich LDL.

1.12.3 Low Density Lipoproteins (LDL)

LDL is the major cholesterol-containing lipoprotein. LDL molecular weight is 2,300,000 Da with the major structural protein being the apo B-100. LDL is formed during the progressive lipolysis of VLDL and IDL. LDL particles are taken up mainly by liver or by extrahepatic tissue via LDL-receptor. LDL is the most important lipoprotein in terms of potential for atheromatous CVD and the availability of effective treatments.

1.12.4 High Density Lipoprotein (HDL)

HDL is synthesized in the liver and also by the intestine and carries apo A-I, A-II, and A-IV. HDL molecular weight is between 67,000 to 360,000 Da. HDL can be divided into subtypes including HDL-2 and HDL-3 depending on the size (Ansell et al., 2005, Havel, 2001). Circulating HDL is able to remove cholesterol from cell surfaces (caveolae) and return it to liver, a process known as reverse cholesterol transport (Figure 1.7). This process is mediated by lecithine:cholesterol acyltransferase (LCAT) and cholesterol ester transfer protein (CETP) (Yokoyama, 2006). CETP is required to facilitate the exchange of cholesterol and TG between HDL and LDL and TG-rich lipoproteins. LCAT plays an important role by esterfication of cholesterol taken up by nascent HDL forming cholesterol ester-rich lipoprotein HDL_2 particles. This process is known as the HDL maturation. Cholesterol ester-rich HDL is large and more spherical (Cho, 2009, Havel, 2001). LCAT is activated mainly by apo A-I. Other factors also play important roles in the transfer of cholesterol such as ATP-binding cassette transporter A1(ABCA1) which is considered the rate-limiting step for HDL assembly (Yokoyama, 2006).

HDL plays an essential part in the metabolism of other lipoproteins. One example is when HDL provides apo C and apo E for hydrolysis of nascent chylomicrons and VLDL (Cho, 2009). Since HDL is considered to be protective, anti-oxidant and anti-atherogenic, its metabolism and potential interaction with adipose tissue is also of interest in ESRD (Ansell et al., 2005, Michael and Peter, 2007).

1.13 White adipose tissue metabolism

It has been known that WAT plays a significant role in the regulation of lipids and glucose metabolism. Any imbalance between storage and mobilization of TGs in WAT will result in disease including obesity and therefore lead to abnormal plasma lipids. Abnormal circulating lipids are a leading causal risk of cardiovascular disease. WAT is the largest organ of energy storage. The synthesis and break down processes of TGs are controlled by hormonal and dietary factors as well as the key enzyme, hormone-sensitive lipase (HSL).

1.13.1 Triglyceride synthesis and storage

1.13.1.1 Synthesis from Fatty Acids

Fatty acids can be obtained either by *de novo lipogenesis* or by uptake from plasma. *De novo lipogenesis* is synthesis of fatty acids from non-lipid molecule mainly glucose. *De novo lipogenesis* is regulated by hormones and nutritional states. These include insulin and poly-unsaturated fatty acids (PUFA). Citrate is converted in the cytoplasm into acetylCoA and oxaloacetate by ATP-Citrate lyase and then into malonyl-CoA. Acetyl-CoA combines with malonyl-CoA to synthesize acyl-CoA by fatty acid synthase (FAS) (Dinel et al., 2010). Figure 1.4 shows lipogenesis pathway in adipocytes. The regulation by insulin and PUFA is

under action of transcription factor sterol response element binding protein 1c (SREBP-1C) and carbohydrate response element binding protein (CHREBP). The regulation of *de novo lipogenesis* in human adipocytes is still not well defined.

Another source of fatty acids is up take from plasma. These fatty acids are circulating in plasma as non-esterified fatty acids bound to plasma albumin or derived by hydrolysis of TG-rich lipoprotein. Human adipocytes express several fatty acid transporters on the plasma membrane; these include the protein CD 36, the fatty acid transport protein, and the fatty acid binding protein plasma membrane (Large et al., 2004, Trayhurn, 2007)

1.13.1.2 Source of glycerol-3-phosphate (G-3-P)

G-3-P is required for synthesis of TG. Since the adipose tissue lacks glycerol kinases, the G-3-P can be produced only from glucose through glycolysis or from gluconeogenesis. Glucose transporters 1 and 4 (GLUT 1 and 4) play important roles for glucose entry into adipocytes (Large et al., 2004).

1.13.1.3 TG synthesis

TGs are produced by a pathway in which G-3-P reacts with fatty acyl CoA to form TG involving many steps. The intermediate products are involved in adipose tissue signaling which regulate many biological processes. Therefore any dysregulation in this pathway may result in a defect of the cellular function (Large et al., 2004).

1.13.2 Lipolysis

Adipose tissue is considered as largest organ in the body for energy storage and a

major source of metabolic fuel. During lipolysis, triglycerides are sequentially hydrolysed into diacylglycerol (DAG), monoacylglycerol, and glycerol, releasing one molecule of fatty acids at each step. Several lipases and lipid droplet-associated proteins such as perilipins are required in the hydrolysis of stored TG (Lafontan and Langin, 2009). Three enzymes are known at the present time involved in the hydrolysis of triglycerides: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) (Girousse and Langin, 2011).

During fasting, lipolysis is activated due to the decreased insulin and increased glucagon concentrations in the post-absorptive states. HSL and other lipases hydrolyze TG to yield free fatty acids, via diacylglycerol and monoglycerol, and glycerol which are released into the blood stream. Fatty acids bound to albumin enter muscles and other tissues to be oxidized for energy while glycerol is converted by the liver to glucose during fasting during gluconeogenesis. HSL and ATGL appear to be the main responsible enzymes in the adipose tissue lipolysis that is controlled by insulin and catecholamines. The coordination of lipid storage and utilization is regulated by the perilipin family of lipid droplet coat proteins (Ducharme and Bickel, 2008). Perilipin A is the most highly phosphorylated protein stimulated by lipolytic hormones in adipocytes and is required for the TG hydrolysis stored in adipocyte lipid droplets by adipocyte lipases (Lafontan and Langin, 2009).

HSL and perilipin phosphorylation is an essential step to activate the lipolysis. In addition, perilipin phosphorylation leads to interact with ATGL which in turn initiate TAG breakdown (Girousse and Langin, 2011, Lafontan and Langin,

2009).

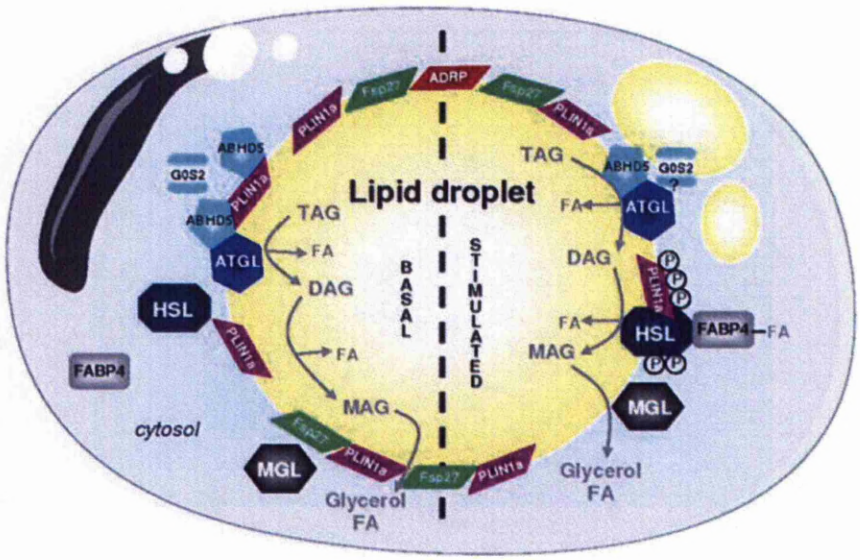

Figure 1.4: An overview of lipid metabolism in white adipose tissue

Adipocyte in basal and stimulated state. In the basal (unstimulated) state, HSL remains in the cytosol while ATGL, perilipin and the ATGL co-activator ABHD5 are located at the LD surface. Perilipin and ABHD5 form a complex, maintaining the latter in an inactive state and thereby limiting basal ATGL-mediated lipolysis. When stimulated, activating pathways trigger protein kinase phosphorylation leading to HSL and perilipin phosphorylation. Phosphorylated HSL migrates from cytosol to the LD where it is enzymaticaly active while LD surface undergoes major rearrangements and fragmentation because of protein kinase-mediated phosphorylation of perilipin. Perilipin phosphorylation is also responsible for ABHD5 release, which can in turn interact with ATGL, to initiate TAG breakdown. ABHD5, abhydrolase domain containing 5; ADRP, adipose differentiation-related protein; ATGL, adipose triglyceride lipase; DAG, diacylglycerol; FA, fatty acid; FABP4, fatty acid-binding protein 4; Fsp27, fat-specific protein 27, also called Cidec; G0S2, G0/G1 switch gene 2; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MGL, monoglyceride lipase; PLIN1a, perilipin A; TAG, triacylglycerol.Adapted from:(Girousse and Langin, 2011).

1.14 Key protein factors in lipid metabolism

1.14.1 Lecithin: cholesterol acyltransferase (LCAT)

LCAT is a plasma enzyme for the esterification of cholesterol to be taken up by HDL. It is expressed mainly in adipose tissue and in small amounts in the brain. Cholesterol esterification is an essential step in the reverse cholesterol transport. LCAT also enhances the HDL maturation from cholesterol-poor to cholesterol-rich particles (Rousset et al., 2009). LCAT requires apo A-I as an activator which is found on the HDL surface. LCAT activity has been noted to be low in ESRD patients (Vaziri, 2006).

1.14.2 Lipoprotein lipase (LPL)

LPL is an enzyme that belongs to the TG lipase gene family of protein that acts as an esterification factor for TG and phospholipase. This family includes hepatic lipase, pancreatic lipase, and endothelial lipase (Wang and Eckel, 2009).

LPL synthesized in adipocytes as well as in other cell types such as myocyte, cardiomyocyte and macrophage (Kobayashi et al., 2007). The main targets of LPL are chylomicrons and VLDL particles as it converts them into remnant forms to facilitate their clearance. Post-heparin plasma is used to measure LPL activity as LPL is detached from the endothelial cells in the presence of heparin (Kobayashi et al., 2007). In ESRD, LPL activity has been reported as low in postheparin lipolytic activity. In addition, Apo C-II which is an LPL activator has also been noted to be low in plasma (Vaziri, 2006).

1.14.3 Cholesterol ester transfer protein

CETP is a transfer protein belongs to a family of protein that enables lipid exchanges in the circulation. This family includes lipopolysaccharide binding protein (LBP), bactericidal/permeability-increasing protein (BPI) and phosphoplipid transfer protein (PLTP) (Quintao and Cazita, 2010). CETP is a 74000 Da secreted mainly by the liver and encoded by CETP gene. CETP is bound to HDL and transfers cholesteryl ester from HDL to other lipoproteins and to IDL in exchange for TG. Thus increased activity of CETP may result in abnormally high TG content in HDL particles (Weber et al., 2010). It has been suggested that the inhibition of CETP can promote the reverse cholesterol transport, a pathway by which excess tissue cholesterol is transported to the liver for excretion (Jain et al., 2007). CETP plasma levels were noted to be increased in ESRD patients (Vaziri, 2006).

1.14.4 LDL-receptor (LDL-R)

The LDL-R is an 839 amino acids transmembrane protein that plays an important role in cholesterol homeostasis. LDL-R takes up cholesterol and cholesrteryl ester from LDL via a process known as clathrin-mediated endocytosis (Gent and Braakman, 2004). LDL-R expression has been studied in many diseases, including ESRD, where it was found that LDL-R expression is associated with heavy proteinuria (Vaziri, 2006).

1.14.5 Acylation stimulating protein (ASP)

ASP is a 76 amino acid protein with molecular weight 8932 Da. ASP is composed of C3desArg, which is produced by adipocytes when adipsin cuts the complement

protein B and C3 complex. C3α results from interaction of C3 with factor B in which adipsin cleaves this interacting complex to generate C3α and C3β chains. Arginine is removed from C3a by carboxypeptidase to form ASP. ASP is produced by adipocytes (Cianflone et al., 2003). Chylomicrons promote secretion of C3 which in turn increases the formation of C3α (Miner, 2004).

ASP stimulates TG synthesis by incorporation of fatty acids into adipose triglyceride. ASP also increases glucose transport (Cianflone et al., 2003). It has been found that ASP plasma levels increased after eating suggesting its role in adipose tissue metabolism and in insulin secretion (Miner, 2004).

1.14.6 Hormone-sensitive lipase (HSL)

HSL is a 775 amino acid enzyme protein produced mainly in adipose tissue among others (Large et al., 2004). HSL has been shown to be an important factor for normal structure and function of adipose tissue (Wang et al., 2008a). HSL is a multifunctional enzyme that hydrolyzes TG, DAG, cholesterol ester and retinyl ester (Lafontan, 2008). $HSL^{-/-}$ models showed an accumulation of DAG in adipocytes and muscle tissue suggesting that HSL is the rate-limiting enzyme for DAG lipolysis (Large et al., 2004). HSL action is regulated by many factors such as adipocyte protein aP2 and fatty acid-binding protein 4 (FABP4) (Lafontan, 2008, Wang et al., 2008a).

1.14.7 Hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase)

HMG CoA reductase is the enzyme that is the rate-limiting step in cholesterol biosynthesis. It converts HMG-CoA to mevalonate that is involved in further steps for synthesis of cholesterol and other lipids (Istvan and Deisenhofer, 2001).

Inhibition of this enzyme results in decreased cholesterol synthesis. For this reason, HMG CoA reductase inhibitors are used as cholesterol-lowering drugs (Jain et al., 2007).

1.14.8 Adipose triglyceride lipase (ATGL)

Adipose triglyceride lipase (ATGL) is one of lipase enzymes. ATGL is 54 KDa also known as desnutrin, encoded by PNPLA2 (patatin-like phospholipase domain-containing-2) (Zimmermann et al., 2004). ATGL hydrolyse the first and rate-limiting step in TG breakdown to DAG and NEFAs. At each step of TG breakdown one molecule of NEFA produced and final step generated NEFA and glycerol, which are released by adipocytes. ATGL is in found abundance in adipose tissue and its expression increases during adipocyte differentiation (Mairal et al., 2006). Studies showed that ATGL- deficiency in mice result in obesity due to TG accumulation in adipose tissue (Haemmerle et al., 2006). ATGL and HSL are the major TG lipases. ATGL plays crucial rules in the lipolysis as it provides DAG to HSL. HSL in turn completes full TG hydrolysis (Lafontan and Langin, 2009).

1.14.9 Perilipin

TG is predominately stored within lipid droplets in adipocytes that are surrounded by a phospholipid monolayer containing various lipid droplet proteins. The perilipin family are groups of proteins belong to the PAT family (Lafontan and Langin, 2009). Perilipin A (PeriA) is the most abundant adipocyte lipid phosphoprotein, which is activated by protein kinase A (PKA) and is involved in the regulating of lipid metabolism in adipocytes by controlling various proteins

(Brasaemle, 2007). Moreover, perilipin A is the most highly phosphorylated protein in adipocytes stimulated by lipolytic hormones and is required for the hydrolysis of TG stored in the fat cell lipid droplets by adipocyte lipases. Absence of perilipin resulted in a reduction in hormone-stimulated lipolysis (Lafontan and Langin, 2009).

1.15 Dyslipidaemia in ESRD

It has been established that ESRD is associated with a characteristic dyslipidaemia. Dyslipidaemia in ESRD consists of both an alteration in function as well as in the structure of lipoproteins (Wanner and Krane, 2002). The management of the ESRD-induced dyslipidaemia is one of the principal therapies to decrease complications such as cardiovascular disease (Prichard, 2003). Generally, dyslipidaemia includes hyper-triglyceridaemia, increased VLDL, increase in plasma concentration of remnant lipoproteins, accumulation of oxidized lipids and lipoproteins, decreased plasma levels of HDL and impaired HDL maturation and function (Vaziri, 2009).

Hypertriglyceridaemia results from an increase in the concentration of apo B-containing lipoproteins. Decreased lipolysis of Chylomicrons and VLDL is attributed to increased apo C-III which in turn inhibits LPL (Kaysen, 2006a). LPL inhibition leads to accumulation of apo B-containing lipoproteins including their transformation into the more atherogenic forms (Vaziri, 2006). Accumulation of remnant chylomicrons and VLDL also results from the decrease in LRP and VLDL receptors (Vaziri, 2009).

Apo B-containing lipoproteins undergo composition alteration along with changes

in concentrations. TG content in VLDL is decreased while the cholesterol content is increased. In contrast to VLDL, LDL is associated with increased TG content and decreased cholesterol (Vaziri, 2006).

ESRD is characterized by normal or near normal levels of plasma cholesterol (Chan et al., 2008). LDL in ESRD is characterized by predominance of small dense LDL particles which have an abnormal content of TG (Vaziri, 2006). These small dense particles are more atherogenic because they are prone to more modifications such as oxidation and also have less binding affinity for their receptors (Vaziri, 2009) (Majumdar and Wheeler, 2000).

Plasma HDL levels have been noted to be decreased in ESRD along with an impairment of HDL maturation (Vaziri, 2006). Apo A-I and apo A-II levels are also decreased and lead to both decreased LCAT activity and decreased HDL (Vaziri, 2009).

The accumulation of remnants lipoproteins in the circulation leads to further modification such as oxidation, glycation or carbamoylation. These modifications increase the half-life, decrease receptor binding and increase the formation of foam cells, factors that promote atherosclerosis progression (Wanner and Krane, 2002).

1.16 Dialysis impact on Dyslipidaemia

Dialysis is one of most effective therapies that are used to decrease 'uraemic toxins' and decrease the uraemic symptoms. The type of dialysis may influence the dyslipidaemia of ESRD (Tsimihodimos et al., 2008).

In haemodialysis, dyslipidaemia includes a significant increase in triglyceride-rich

lipoproteins, decrease in HDL and predominance of small dense LDL particles. Circulating LDL-cholesterol or total cholesterol levels are normal or near normal (Chan et al., 2008). There are increases in Lp(a) (Prichard, 2003). In addition, decreased apoC-II/apoC-III ratio leads to low activity of LPL and therefore impaired catabolism of TG-rich lipoproteins (Chan et al., 2008).

In contrast to haemodialysis, peritoneal dialysis seems to be characterised by a more atherogenic lipid profile (Chan et al., 2008, Prichard, 2003, Tsimihodimos et al., 2008). Dyslipidaemia in peritoneal dialysis is characterized by an increase in levels of plasma cholesterol, TG, IDL,VLDL, LDL and Lp(a) (Chan et al., 2008). Patients with peritoneal dialysis also have more susceptibility to insulin resistance as well as more VLDL production due to glucose loading from peritoneal dialysate (Chan et al., 2008, Tsimihodimos et al., 2008).

1.17 Consequence of dyslipidaemia

The risk of CVD is well established in ESRD patients. One of the major consequences of dyslipidaemia in ESRD is cardiovascular disease. Several studies have documented that LDL and HDL cholesterol are risk factors for cardiovascular disease in ESRD (Chan et al., 2008). Dyslipidaemia also promotes the progression of kidney disease. The reabsorption of lipids by tubular epithelial cells may result in the structural and functional changes in the kidney tissue (Chan et al., 2008, Vaziri, 2006). Dyslipidaemia has also been suggested to alter energy metabolism by limiting the availability of fatty acids in the skeletal muscles and myocardium (Vaziri, 2006).

1.18 Management of dyslipidaemia

It has been recommended that dyslipidaemia should be managed in ESRD as it is associated strongly with cardiovascular disease (Marrs and Saseen, 2010). Reduction of total and LDL cholesterol has been shown to significantly decrease the risk of cardiovascular disease in early stage of CKD (Molitch, 2006). Dyslipidaemia can be managed by changing the life styles of patients as well as by pharmacological intervention. The common pharmacological treatments include HMG CoA reductase inhibitors (statins), fibrates and cholesterol absorption inhibitor (Chan et al., 2008). Statins and fibrates have to be used cautiously in ESRD, at reduced doses, as suggested in a recent publication (Kasiske et al., 2006).

The benefit of statins on the prevention of coronary artery disease is well-established (Nair et al., 2010). Statin have also been suggested to have additional effects such as anti-inflammatory effect as seen by a drop in the C-reactive protein (Montague and Murphy, 2009). ESRD patients on statin have been shown to have low mortality when compared with non-statin patients. However, some studies fail to show any benefit from using statin in ESRD (Wanner and Ritz, 2008).

Fibrates are used to reduce plasma triglyceride and increase HDL-cholesterol. Fibrates are agonists of the enzyme peroxisomes proliferator activated receptor alpha (PPARα) which results in the lipolysis of TG-rich lipoproteins due to stimulation of lipoprotein lipase, inhibition of apo C-III secretion, and by increasing the apo A-I production (Jain et al., 2007). Some studies recommend against using fibrate in ESRD whereas others suggest that they be used by specialists cautiously (Kasiske et al., 2006, Montague and Murphy, 2009).

1.19 Rationale for the study of adipose tissue

The adipose tissue is crucially involved in the production and clearance of lipoproteins. This involves a close interaction between the lipoproteins and adipocytes. Understanding how this process is altered in ESRD is critical. It is also tempting to postulate that the lipoprotein/adipocyte interaction may also influence the other functions of adipose tissue including its endocrine function. The present studies are designed to clarify this hypothesis.

Inflammation and obesity in ESRD are independently associated with pro-atherogenic dyslipidaemia (Axelsson, 2008). Therefore studying the adipokine secretion, both pro- and anti-inflammatory, in the context of ESRD and renal replacement therapy is important.

It is also possible that the nutritional and the endocrine functions of adipose tissue may overlap. An association between low plasma adiponectin and decreased lipoprotein lipase activity has been described (Kwan and Beddhu, 2007).

The influence of renal replacement therapy such as dialysis on long term prognosis is not fully understood. Dialysis does not improve the nutritional function of adipose tissue since it has been shown not to correct dyslipidaemia (Majumdar and Wheeler, 2000). The type of dialysis may also be critical since PD patients have a more atherogenic lipid profile than HD patients (Prichard, 2003). Circulating adipokine concentrations are altered in ESRD (Wiecek et al., 2002).

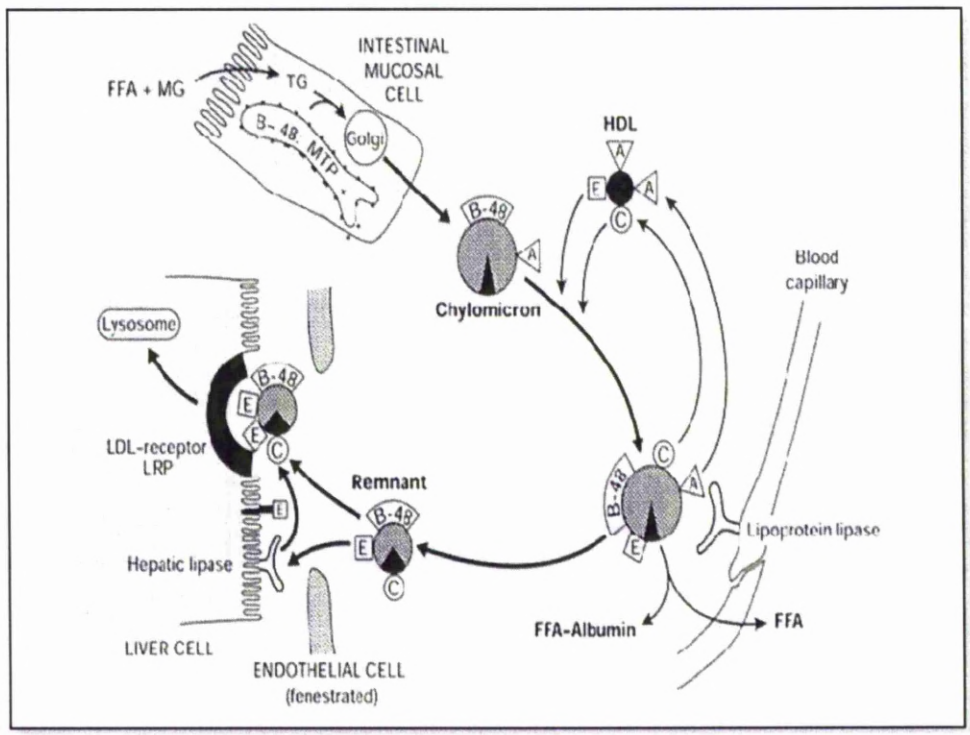

Figure1.5: Chylomicron metabolism

Chylomicrons are synthesized in the intestinal epithelial cells. After secretion CMs interact with HDL where apo C and apo E are transferred from HDL. On the luminal surface of capillary endothelium of adipose tissue, LPL hydrolyzes the TG and remnant CM is produced. The remnant CM is taken up by the liver receptor-mediated endocytosis.

Adapted from (Havel, 2001)

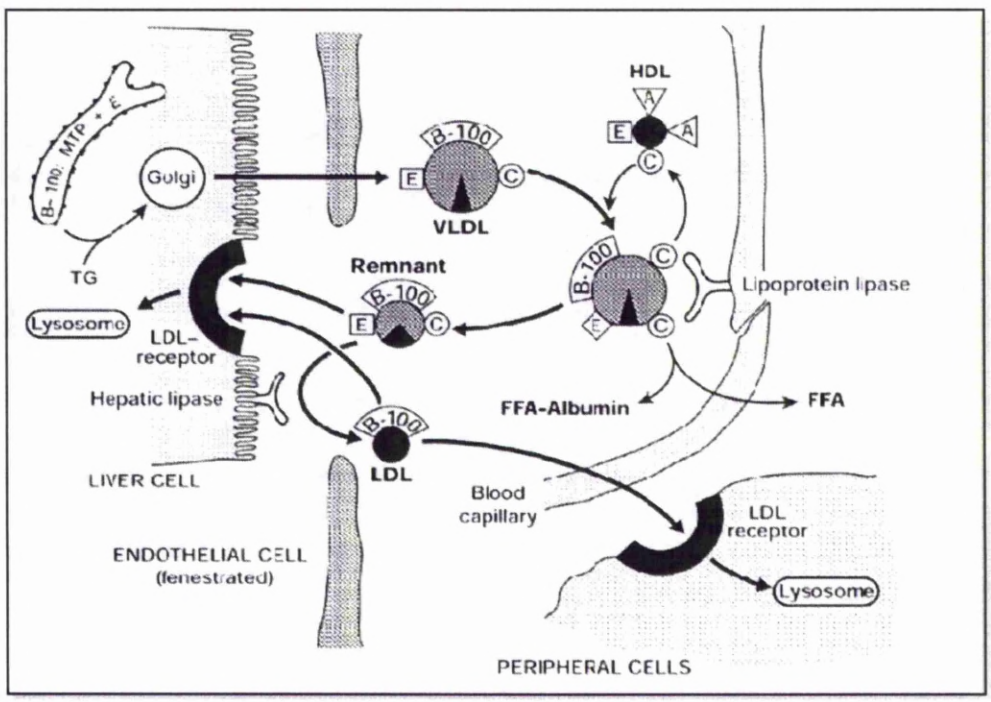

Figure1.6: VLDL and LDL metabolism

Following secretion of VLDL, LPL hydrolyzes the TG forming IDL. IDL can either be endocytosed by the hepatocytes through a receptor-mediated process or undergo further hydrolysis by hepatic lipase leading to formation of LDL. LDL may be taken up by scavenger receptors on non-hepatic cells including macrophages.

Adapted from (Havel, 2001)

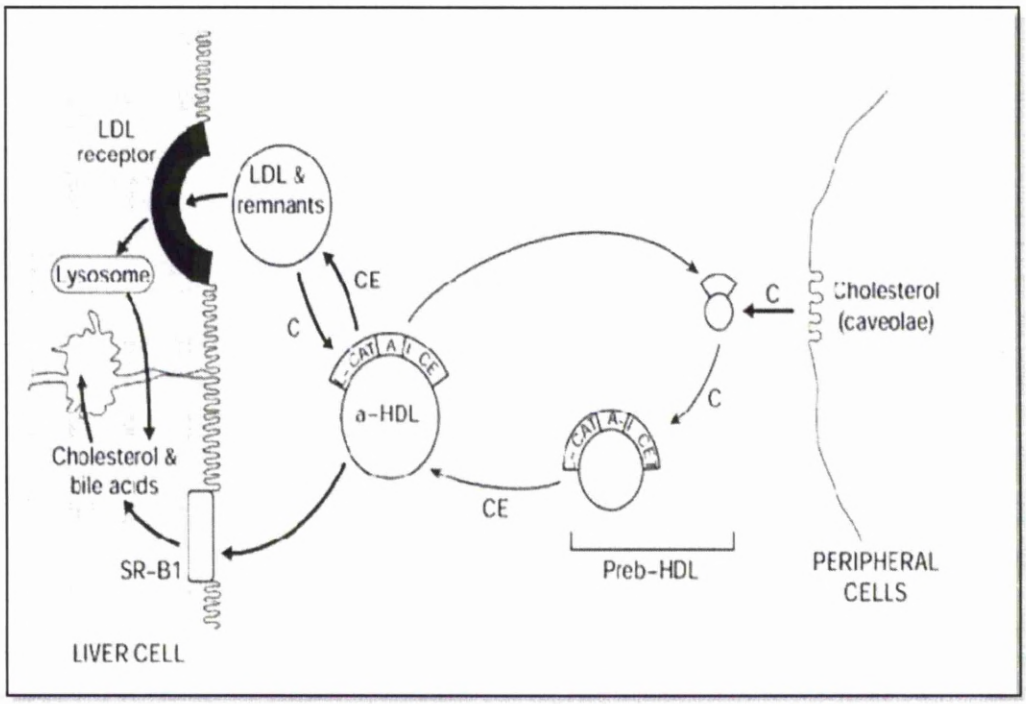

Figure 1.7: HDL metabolism

HDL picks up cholesterol from cells. Cholesterol is converted to cholesterol ester by LCAT. HDL transports CE to LDL or TG-rich lipoprotein by CETP. HDL particles bind to scavenger receptor SR-B1, transferring the cholesterol and cholesterol esters into hepatic cells.

Adapted from (Havel, 2001)

1.20 Novelty of studies in this thesis

Previous studies in this area have largely dealt with circulating adipokine and lipoprotein measurements in ESRD and following dialysis. As far as I am aware this is the first attempt to investigate circulating lipoprotein fractions in ESRD and renal replacement therapies such as HD and PD. A recent previous study has examined 3T3-L1 (mouse) adipocyte response to uraemic serum in a very limited way in terms of a single adipokine, leptin (Kalbacher et al., 2011). This is the first study to attempt to characterise a full range of lipoprotein gene and protein changes brought about by incubation of uraemic serum with normal human adipocytes *in vitro*. For the very first time this study has attempted to characterise a fuller range of changes in adipokines by incubation of uraemic serum with normal human adipocytes. This is also a novel approach to attempt to clarify the effect of renal replacement therapies in their ability to alter lipoprotein metabolism and adipokine secretion in terms of changes in gene and protein expression.

1.21 Hypothesis

Adipose tissue has been shown to contribute to CVD in various ways that include alteration in lipoprotein metabolism as well as secretion of pro- and anti-inflammatory adipokines. We hypothesized that the retention of 'uraemic toxins' in ESRD results in adipose tissue dysfunction affecting both adipokine production and lipoprotein metabolism. This could be a potential link to CVD in ESRD. This could also explain why ESRD patients are relatively resistant to the beneficial effects of statins.

1.22 Aims and objective of study

The objective of the study was to clarify the role that adipose tissue and lipoproteins may have in ESRD. This in turn may increase our understanding of the cardiovascular risk associated with ESRD.

More specifically, we aim to:

i) Determine whether the effects of *serum* from normal and ESRD subjects on adipocyte cultures is different;

ii) Understand how the responses to serum obtained prior to and following dialysis influence adipocyte cultures;

iii) Clarify the effect of chylomicrons, VLDL, LDL and HDL isolated from plasma of *normal* subjects on adipocyte cultures;

iv) Examine the effect of chylomicrons, VLDL, LDL and HDL isolated from plasma of *ESRD* subjects on adipocyte cultures;

v) Study nutrient and adipokine aspects of adipocyte function both in adipocyte cultures and adipose tissue samples obtained from patients with ESRD;

vi) Show whether the adipose tissue taken from ESRD patients is different morphologically from a normal group.

Providing answers to these questions may contribute to a better understanding of the role adipose tissue in the pathogenesis of CVD in ESRD.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents and Methods

2.1.1 Chemical reagents

Acetic acid	Sigma
Agarose	Sigma
Amphotericin B	Lonza
Biotin	Sigma
Bovine serum albumin	Sigma
Chloroform	Fisher
Copper (II) sulphate solution	Sigma
Cortisol	Sigma
Dexamethasone	Sigma
Disodium hydrogen phosphate	Sigma
Modified Eagle's medium/Ham's F12 (1:1)	Invitrogen
100% Ethanol	Sigma
Endothelial cell growth supplement	PromoCell
Epidermal Growth Factor	PromoCell
Ethidium bromide	Sigma
Foetal calf serum	Biosera
Gill's haematoxylin	Sigma
GeneRuler TM 100 bp DNA Dulbecco's ladder	Helena BioSciences
Goat anti-mouse conjugated to horseradish peroxidase	Bio-Rad
Guanidine hydrochloride	Sigma
HEPES	Sigma
Human transferrin	Sigma
I-methyl-3-isobutylxanthine (IBMX)	Sigma
Insulin	Sigma
Isoprenaline Isopropanol	Sigma
Magnesium sulphate	Sigma
Methanol	VWR
Monoclonal Mouse Anti-Human CD68	DakoCytomation

Noradrenaline	Sigma
Normal Goat serum	Vector
Optiprep	Axis-Shield,UK
Pantothenate	Sigma
Penicillin/Streptomycin	Invitrogen
Photographic developer	Sigma
Photographic fixer	Sigma
Potassium chloride	Sigma
Preadipocytes , Human subcutaneous white	PromoCell
RNase AWAY	Molecular BioProd
Rosiglitazone	GlaxoSmithKline
Sodium chloride	Sigma
Sodium citrate	Sigma
Sodium dodecyl sulphate	Sigma
Sodium dihydrogen orthophosphate	Sigma
Sodium EDTA	Sigma
Sodium hydroxide	Sigma
10xTBE buffer	Fisher
Trichloroacetic acid	Sigma
Triiodothyronine (T3)	Sigma
Tris-Base	Sigma
Tris-hydrochloride	Sigma
Trizol	Invitrogen
Trypsin/EDTA	Invitrogen
Tween 20 (polyoxyethylene sorbitan monolaurate)	Dakocytomation
Ultra-pure water	Sigma

2.1.2 Assay Kits

DNA-free Kit	Ambion
ECL detection reagents	Amersham
Human Adiponectin ELISA	R&D System

Human Leptin ELISA	RayBio
Human Interleukin-6	PeproTech
iScript cDNA Synthesis Kit	Bio-Rad
QPCR Core Kit	Eurogentec
Eurogentec	Abgene

2.1.3 Equipment

2011 Macrovue UV transilluminator	Ultra-Violet Products
Beckman Preparative Ultracentrifuge	Beckman
Benchmar Plus microplate spectrophotometer	Bio-Rad
Biophotometer	Eppendorf
Centrifuge 5415 D	Eppendorf
Centrifuge 5415 R	Eppendorf
Coming filter system (0.22 μm membrane)	Fisher
FC205 fraction collector	Gilson
Histocenter	Shandon
Horizontal Gel Electrophoresis units	SCIE-PLAS
Kodak Digital Science DC 120 digital camera	Kodak
Labconco Auto Densi-flow density gradient fractionator	Labconco
AS 325 Microtome	Shandon
Microscope ((Polyvar)	Leica
Mx3005PTM QPCR System	Stratagene
PCR Express thermal cycler	Hybraid
PhotoEnhancer	Kodak
3310 pH meter	Barloworld
Processor 2LE	Shandon
Quick-seal tube (for rotor VTi 65.1)	Beckman
L8-80 Ultracentrifuge	Beckman
Roche Modular™ SWA	Roche Diagnostics GmbH
UVette® plastic disposable cuvettes	Eppendorf
Water bath	Grant
12-well plate	Fisher

48-well plate	Fisher
96-well microplate	Fisher

2.1.4 Software

GraphPad InStat version 5	GraphPad
Kodak Digital Science ID 1 image analysis software	Kodak
Microplate Manager v5.2	Bio-Rad
MXPro^{TM} QPCR Software	Stratagene

2.2 Blood and Tissue samples

2.2.1 Subjects

18 male patients with ESRD on maintenance haemodialysis and peritoneal dialysis were recruited on this study. Patients diagnosed with diabetes mellitus were excluded where possible. The medical records of each participant were reviewed carefully as regards demographic data and underlying diseases such as coronary artery disease, hypertension, diabetes mellitus as well as medication history. The study protocol was approved by Liverpool Research Ethics Committee (REC number: 08/H1002/41). All patients gave informed consent. Inclusion criteria consisted of ESRD patients on dialysis, and exclusion criteria were age >70 years, female patients, diabetic whenever possible and unwillingness to participate in the study. Most patients were on antihypertensive medications as well as other commonly used drugs in ESRD, such as phosphate and potassium binders (sodium polystyrene sulphonate), diuretics, and vitamin B, C, and D supplementation (full list on appendix 2).

The enrolled subjects were categorized into 3 groups: (i) 9 subjects (age: 45.4 ±

8.1 years) without CKD as controls and without any evidence of kidney injury, (ii) 11 patients (age: 47.5 ± 5.5 years) prior to starting their dialysis as Pre-haemodialysis group (Pre-HD), (iii) the same 11 Pre-HD patients (age: 47.5 ± 5.5 years) following their dialysis as Post-haemodialysis group (Post-HD) and (4) 7 patients (age: 48 ± 14.2 years) on peritoneal dialysis as peritoneal dialysis group (PD).

2.2.2 Haemodialysis procedure

Haemodialysis (HD) is a method for removing waste products such as creatinine and urea, as well as free water and other toxins from the blood when the kidneys are in renal failure. HD is one of three renal replacement therapies (the other two being renal transplant and peritoneal dialysis). HD in the studies described in this thesis was carried out as an outpatient therapy on the renal wards of the RLUH. HD treatments were initiated and managed by specialized staff made up of doctors, nurses and technicians on the renal wards at the RLUH.

The principle of HD is the same as other methods of dialysis involving diffusion of solutes across a semipermeable membrane. HD utilizes counter current flow, where the dialysate is flowing in the opposite direction to blood flow in the extracorporeal circuit. Counter-current flow maintains the concentration gradient across the membrane at a maximum and increases the efficiency of the dialysis. Fluid removal (ultrafiltration) was achieved by altering the hydrostatic pressure of the dialysate compartment, causing free water and some dissolved solutes to move across the membrane along a created pressure gradient. The dialysis solution that

was used was a standard sterilized solution containing mineral ions (HCO_3, Ca^{2+} and K^+ and Na^+).

Eleven patients with ESRD were identified as being suitable for the study following an initial screening of 51 patients. These patients were stable and receiving HD three times a week. Each HD session lasted 4 hours. All patients had good vascular access due to presence of a fully functioning arteriovenous fistula. All HD patients received a loading dose of 1000 IU of unfractionated heparin at the start followed by 1000 IU per hour during haemodialysis. All patients had their vital signs including blood pressure and pulse monitored during and after dialysis. Euvolaemia was achieved in all patients post-HD.

2.2.3 Peritoneal Dialysis (PD)

Peritoneal dialysis uses the peritoneum as a natural permeable membrane through which water and solutes can equilibrate. PD is less physiologically stressful than HD, does not require vascular access, can be done at home, and therefore allows patients much greater flexibility. However, it requires much more patient involvement. Of the total estimated resting splanchnic blood flow of 1200 mL/min, only about 70 mL/min comes into contact with the peritoneum, so solute equilibration occurs much more slowly than in HD. But because solute and water clearance is a function of contact time and PD is done nearly continuously, efficacy in terms of solute removal is comparable to that obtained with HD.

Dialysate in the PD carried out in the 7 patients in this thesis was instilled through a catheter into the peritoneal space, was left to dwell, and then drained. In the double-bag technique, the patient drains the fluid instilled in the abdomen in one

bag and then infuses fluid from the other bag into the peritoneal cavity. Both bags are removed between dwells in what is known as the 'disconnect' system. Continuous ambulatory peritoneal dialysis (CAPD), used in all 7 of the patients in this thesis, is most commonly used because of ease of performance and lack of need for a machine to do the exchanges. Typically 2 to 3 L of dialysate was infused 4 to 5 times/day; dialysate is allowed to remain for 4-5 h during the day and 8 to 12 h at night. The solution was manually drained. Heparin is not required and was not administered for this group of patients.

2.2.4 Blood

Blood was collected from all participants, before and after dialysis for HD group, and once for the other groups. 30 ml of blood samples from each were transported to the Clinical Biochemistry Department at RLUHT and centrifuged at 3000 g for 15 min followed by separation of serum or plasma. Samples were aliquoted and stored at – 80°C until analysis.

2.2.5 Preparation of pooled samples

Individual patient samples were analysed and reported in result chapters. However in chapters 4 to 6, pooled serum was used. Pooled serum was prepared by adding equal volumes taken from each patient blood sample immediately after collection. The objective of pooling was to study the group as a whole in terms of disease or health and type of therapy in general rather than to isolate individual patient differences. This strategy was necessary for practical purposes. Otherwise the workload would have been too great making it impossible to complete the studies and beyond the scope of this thesis.

2.3 Biochemical analysis

Urea, Creatinine, cholesterol, TGs, LDH and total protein concentrations were measured on a Roche Modular™ SWA analyzer using colorimetric or enzymatic assays following the fully validated assay procedures in routine use in the department of Clinical Biochemistry (Roche Diagnostics GmbH, Mannheim, Germany). LDL-cholesterol was calculated from total cholesterol, and HDL-cholesterol and TG by means of the Friedewald formula;

LDL cholesterol = Total cholesterol – HDL cholesterol – Total triglyceride ÷ 2.19

2.3.1 Principle of cholesterol measurement

Total cholesterol concentrations were determined enzymatically. In the reaction the cholesterol esterase hydrolyzes cholesterol esters to free cholesterol and fatty acids. The free cholesterol is oxidized to cholesten-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalyzes the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a colored quinoneimine product. The system monitors the change in absorbance at 520 nm at a fixed-time interval. The change in absorbance is directly proportional to the concentration of cholesterol in the sample.

2.3.2 Principle of Triglyceride measurement

Total TG concentrations were determined enzymatically in two steps: Briefly, free glyceride, was first converted to glycerol 3-phosphate by glycerol kinase and degraded into dihydroxyacetone 3-phosphate plus hydrogen peroxide by glycerol-3-phosphate oxidase. The hydrogen peroxide was further degraded into H_2O and O_2 by peroxidase. In the second step, TG was degraded into glycerol by

lipoprotein lipase in surfactant, followed by the same reactions as in the first step. The generated hydrogen peroxide was measured quantitatively by colorimetric assay. The reaction is measured at 505 nm. The change in absorbance is directly proportional to the concentration of TG in the sample.

2.3.3 Principle of HDL-cholesterol measurement

In this method water-soluble complex with non-HDL cholesterol fractions is formed by adding magnesium/dextran sulfate solution to the specimen. In next step, HDL-cholesterol esters are converted to HDL-cholesterol by PEG-cholesterol esterase. The HDL-cholesterol is acted upon by PEG-cholesterol oxidase, and the hydrogen peroxide produced from this reaction combines with 4-amino-antipyrine and HSDA under the action of peroxidase to form a purple/blue pigment that is measured at 600 nm.

2.4 Lipoproteins separation techniques and measurement

Plasma lipoproteins can be separated by many techniques depending on their physical properties. These techniques including ultracentrifugation, sequential precipitation, size exclusion chromatography, affinity chromatography and gel electrophoresis (Wasan et al., 2000). Some of these techniques are not used as routine methods in the diagnostic laboratories because they are time-consuming. However, ultracentrifugation technique is considered the most acceptable technique for its ability to separate the major lipoproteins and their subclasses reproducibly despite limitations in equipment availability (Graham et al., 1996, Wasan et al., 2000). In this thesis, a method was developed by Graham et al (Graham et al., 1996) using iodixanol density gradient centrifugation to separate

lipoprotein fractions. This method was performed by using iodixanol which was originally used as an X-ray contrast medium due to its relative nontoxicity (Graham et al., 1994).

Electrophoresis is another widely used technique in the clinical laboratories where lipoprotein classes can be separated on cellulose acetate, agarose and polyacrylamide gel. Separated lipoproteins can be quantified or semi-quantified by further steps such as estimation of their mass or lipid content. However, measurement of cholesterol in the lipoprotein particles, such as HDL and LDL, is the most widely used measurement in the diagnostic laboratories.

2.4.1 Lipoproteins fractionation

The main classes of plasma lipoprotein, very low density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL), can be separated depending on their differences in density and charge. Separation of lipoproteins into different fractions in this study was performed by the use of self-generating continuous gradients of iodixanol (Graham et al., 1996). Iodixanol is a nontoxic and inert compound capable of providing reliable and reproducible experimental results (Ford et al., 1994).

2.4.2 Plasma Preparation

Blood was centrifuged at 2000 *g* for 10 min to pellet the leukocytes and erythrocytes. The supernatant plasma was transferred to a conical centrifuge tube; 0.3 mL of phosphate buffered saline (PBS) was layered on the surface, followed by centrifugation at 13000 *g* for 20 minutes when chylomicrons floated to the surface. The resultant chylomicron-containing layer on the surface was removed

using an automatic pipette.

2.4.3 Generation of Iodixanol gradients

4 mL chylomicron-free plasma was added to 1mL of 60 % (W/V) iodixanol. 5 mL of 6% (W/V) iodixanol in PBS was placed in a 13x35 mm polyallomer centrifuge tube (Optirprep). The iodixanol-plasma was carefully layered underneath the 6 % (w\v) iodixanol solution. Approximately 1 mL of PBS was then added to fill the tube and subjected to centrifugation at 65,000 RPM, at 16°C for 190 min (VTi 65.1 rotor , Beckham L8-80 ultracentrifuge)(Figure 2.1).

2.4.4 Collection of plasma fractions

All tubes were placed in the gradient unloader and the fractions were collected using an automated fractionator (autodensi-flow 451–7200, Labconco). 1 mL aliquots were initially removed from the top of each tube and transferred to a microfuge tubes. The aliquots were stored at -70 C until further analysis in order to identify the fraction, each fraction then assayed for the concentrations of triglyceride, cholesterol and total proteins.

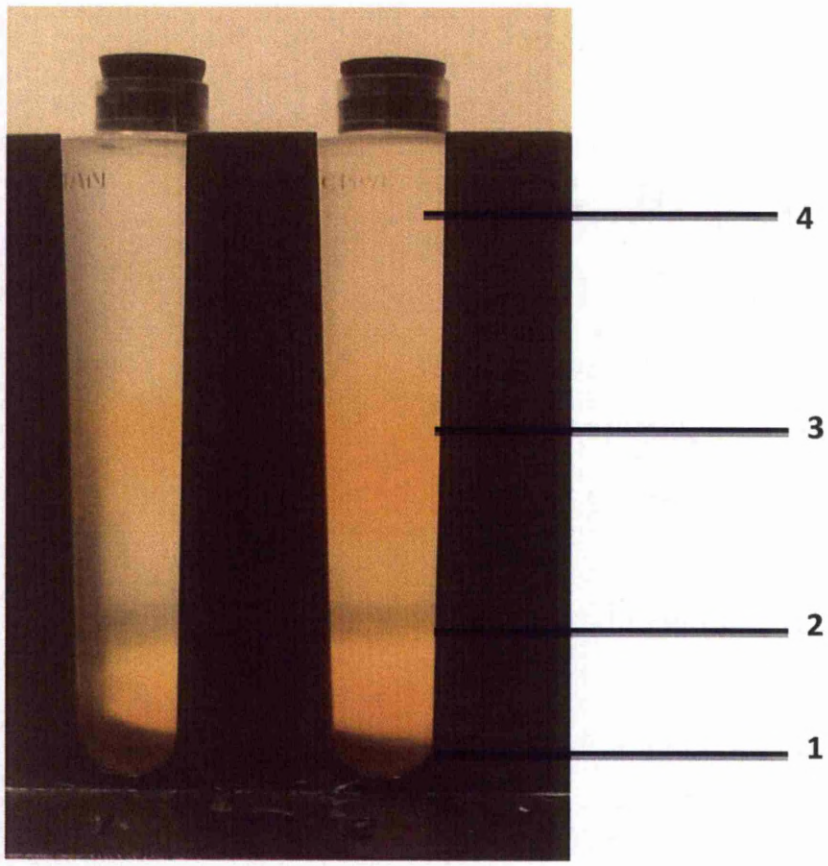

Figure 2.1 Separation of plasma lipoproteins in self-generated gradients of iodixanol

Plasma was separated in a self-generated gradient of iodixanol. The gradients were collected and a sample from the fraction containing the peak VLDL, LDL, and HDL was analysed for cholesterol and protein. A representative centrifuge tube illustrating the separation is shown: 1, Plasma protein; 2, HDL; 3, LDL; 4, VLDL.

2.5 Adipose tissue biopsy and histological procedures

In order to examine white adipose tissue by light microscopy samples underwent fixation to maintain the ultrastructure features of the cells to allow accurate morphometric assessment. Tissue sections from different depots of fat (subcutaneous and visceral) were carefully dissected, divided into 1 cm^2 portions, fixed for 18 h and prepared using the following methods.

2.5.1 Biopsy

Patient tissue samples of omental adipose (n=6) and subcutaneous (n=6) tissue had been collected with informed consent during kidney transplantation or other open surgery. Adipose samples from the non-renal failure patients (healthy donors), namely omental adipose (N=6) and subcutaneous (N=6) tissue samples were also collected and used as controls. The study protocol was approved by Liverpool Research Ethics Committee (REC number: 08/H1002/41). Samples were placed in fixation reagent immediately upon collection.

2.5.2 Fixation

Immediately after dissection, tissue samples were placed in freshly prepared 4% paraformaldehyde (PBS buffer, pH 7.6) for 4-8 hours depending on the biopsy sizes, as a guide, 1 cm^2 portions were fixed for 18 h. Samples were processed to form paraffin blocks using a Shandon Citadel 2000 automatic processor,

2.5.3 Dehydration

As the embedding materials are water insoluble, it is necessary to completely remove tissue associated water to allow maximum infiltration by the resin. The

samples were serially dehydrated in increasing concentrations of ethanol as follows:

i) 15%, 30%, 50% ethanol, 2 changes, 30 minutes each

ii) 70% ethanol overnight

iii) 80%, 90%, 95% ethanol, 2 changes, 30 minutes each

iv) 100% ethanol, 6 changes, 30 minutes each

2.5.4 Embedding to paraffin wax blocks

This process allows the tissue to be infiltrated uniformly. The tissue samples were placed in metal moulds, and coded wax added being careful to avoid bubble formation.

2.5.5 Sectioning

The hardened wax-embedded tissue blocks were trimmed with a sharp razor blade to remove excess wax. Knives mounted on a microtome were used to cut the blocks down to the level of tissue. Once the block surface was smooth a new knife was used to cut and collect 0.5 μm sections. The sections were collected using metal forceps, placed on a drop of water on a glass slide, and stretched using a cotton stick immersed in trichloroethylene. Sections were left to dry on a hot plate for 5 min approximately then mounted on glass microscope slides.

2.5.6 Haematoxylin and Eosin staining

Sections were dewaxed in xylene for 15 min and rehydrated through a series of decreasing ethanol concentrations from 100% to 90% 2 changes 5 min each.

Slides were then placed in Gill's haematoxylin for 1-2 minute and washed in water. Increasing concentrations of ethanol from 90 to 100 % were used to dehydrate the tissue sections and xylene was used to clear the sections for 15 min. Sections were then mounted with DPX.

2.5.7 Light Microscopy

From each biopsy one suitable section was selected and photographed using a digital Kodak camera attached to a photo light microscope. Sections were photographed at 2 magnifications (x20 and x40)

2.6 Immunohistochemistry

2.6.1 Sample fixation

Tissue samples were immediately fixed in freshly prepared 4% paraformaldehyde in PBS buffer pH 7.6 for 4-8 hours depending on the biopsy sizes. Samples were processed to paraffin blocks as described in sections 2.5.1 to 2.5.5.

2.6.2 Cutting

Sections of adipose tissue were cut using a microtome. 5μm ribbons of paraffin-embedded tissue were cut with a microtome in accordance with the manufacturer's instructions. The ribbon was divided into three section pieces by using the scalpel then transferred to the 50°C transfer bath.

2.6.3 Mounting

Sections were mounted on positively charged glass slides using a water bath maintained at 45° C and allowed to dry in an oven for several hours at 60° C.

Day 1

2.6.4 Dewaxing

Tissue sections were dewaxed in xylene by immersing the slides for 3 x 10 min each in a xylene bath and then two times for two min each in a reducing concentration of alcohol baths (100%, 95%, 90%, 70%) followed by rehydration in distilled water. The slides were rinsed with Tris-buffered saline in 0.01% Tween 20 (TBS/Tween20) for five min on an orbital shaker to enhance antibody penetration.

2.6.5 Antigen retrieval by microwaving method

30 ml of 0.1 M sodium citrate pH 6 was added in a slide bath and covered with a loosely fitting lid. The slide bath was placed inside a larger container with 600 ml of distilled water and both containers were placed inside a microwave oven (900W) and heated at full power, then incubated for 20 min. The slide bath was allowed to cool for 20 min and the slide washed 4 times in PBS.

2.6.6 Endogenous peroxidase blocking

A solution of 3% hydrogen peroxide was used to block endogenous peroxidase activity. The slides were incubated in blocking solution on an orbital shaker at room temperature for 30 minutes, rinsed 3 times in distilled water and transferred to TBS/Tween20 for 10 min.

2.6.7 Blocking non-specific staining

After encircling the area around the sections using a Pap-Pen, a 1:10 solution of normal non-immune serum, in which the secondary antibody was raised, was

added and the sections were incubated in a humidified chamber for 30-45 min. This step prevents background staining by blocking non-specific binding of antibodies to the tissue. Before the application of the primary antibody, excess serum was removed from the slides by gentle tapping.

2.6.8 Primary antibody

Tissue sections were incubated with the primary antibody in a humidified chamber at 4°C overnight. The dilution of primary antibody was established through experimental trials, starting with the initial dilution suggested by the manufacturers. Primary antibodies were diluted in Tris HCl containing 1% BSA

Day 2

Between each step, tissue sections were washed in TBS/Tween three times for 5 min each.

2.6.9 Secondary antibody

Biotin conjugated secondary antibody was applied at the recommended dilution in the appropriate non-immune serum in Tris HCl/1% BSA. Samples were incubated in a humidified chamber for 45 min on an orbital shaker at room temperature and developed using 3, 3'-Diaminobenzidine (DAB) chromogen.

2.6.10 Counter Stain

Slides were counterstained by Gill's haematoxylin for 4 minutes.

2.6.11 Dehydration

The sections were dehydrated in alcohol two times for 1 min each, followed by

xylene three times for 2 min each.

2.6.12 Mounting

The sections were mounted with DPX and cover slips placed over them.

2.7 Adipocyte cell Culture (Promo Cell)

2.7.1 Reagents

2.7.1.1 Pre-adipocyte medium

DMEM/Nutrient Mix F12

5 % FCS

9.9 µg/ml Endothelial cell growth supplement

200 µg/ml EGF

1µg/ml Hydrocortisone

100 units/ml Penicillin

100 µg/ml Streptomycin

0.25 µg/ml Amphotericin B

2.7.1.2 Induction Medium

DMEM/Nutrient Mix F12

32 µM Biotin

100 nM Human insulin

1 µM Dexamethasone

200 µM IBMX

10 mg/ml Rosiglitazone

11nM L-Thyroxine

100 units/ml Penicillin

100 µg/ml Streptomycin

0.25 µg/ml Amphotericin B

2.7.1.3 Feeding Medium

DMEM/Nutrient Mix F12

5 % FCS

32 µM Biotin

100 nM Human insulin

1 µM Dexamethasone

100 units/ml Penicillin

100 µg/ml Streptomycin

0.25 µg/ml Amphotericin B

2.7.1.4 1x PBS

0.286 M sodium chloride

5.55 mM potassium chloride

16.4 mM disodium hydrogen phosphate

2.94 mM potassium dihydrogen orthophosphate

Autoclaved distilled water

2.7.1.5 Trypsin/EDTA

Autoclaved distilled water

0.005%/0.002% trypsin/EDTA

1x PBS

All media were prepared using a sterilizing filter system in a cell culture laminated air flow hood. Media was stored at 4°C and warmed to 37°C prior to use. The culture and feeding media was used within 2 weeks. The induction medium, however, must was made immediately before use.

2.7.2 Cell recovery

Human white preadipocytes (Promo Cell, UK), stored in cryovial were removed from the liquid nitrogen and quickly thawed in a 37°C water bath. The thawed cell suspension was transferred to a 75 cm^2 flask containing 20 ml of preadipocyte culture medium. Cells were maintained for approximately 6 days with one change of medium or when they had reached 80%-90% confluency. The cells were maintained in an incubator at 37°C in a humidified atmosphere with 5% CO_2/95% air. At this point, the cells were ready to be passaged and subcultured. Prior to trypsinisation, the cells were briefly washed once with sterilized 1x PBS. Then, 9 ml of trypsin/EDTA was added to the flask and placed at 37°C for 1 min to detach the cells. Cell detachment was maintained by light microscopy. After the cells were entirely detached, 9 ml of culture medium was added in a final volume of 10 ml. The cell suspension was mixed by sterile pipettes several times. An equal density of preadipocytes was seeded into new flasks, and 12-well or 48-well plates according to the experimental requirement.

2.7.3 Cell induction

Differentiation of the cells was initiated 24 h after plating. Following

aspiration of the old medium, the cells were gently washed with pre-warmed 1x PBS, three times and then incubated for 4 days in the FCS-free induction medium. Differentiation into adipocytes was examined under a light microscope by phase contrast at 100x magnification. The phase contrast image of the accumulation of lipid droplets during the development of adipocytes was captured and photographed by a CCD video camera (UVP Inc., California, USA) (Figure 2.2). More than 90% of Promo cells underwent full differentiation into mature adipocytes after day 10 post-induction.

2.7.4 Cell Treatment

To examine the adipokine gene expression and secretion, fully differentiated adipocytes were incubated with serum or lipoprotein fractions; at day 12-14 post-induction for 4 and 24 h. Cells incubated in adipocyte medium without added serum or lipoprotein fractions for the same period were used as controls. Incubations at each time-point were performed in replicates of 4 or 6.

2.7.5 Harvesting the Cells

According to the particular experimental design, the cells were collected at several time-points in 700 µl of Trizol per well. Samples were stored at -80°C until analysis.

2.7.6 Cytotoxicity study

At the end of the exposure period, the cytotoxicity was evaluated by lactate dehydrogenase (LDH) release and by examining microscopically the cell shape and integrity (Roche, Germany). The culture medium was harvested for the measurement of LDH activity. The LDH activity of the medium and cell lysate

was determined by spectrophotometric monitoring of the decreasing level of NADH described in the following reaction:

Pyruvate + $NADH+H^+$ ----→ Lactate + NAD^+

The following formula was used to calculate cytotoxicity:

Cytotoxicity (%) = (experimental value - low control)/high control – low control)*100

Low control was defined as the sample containing assay medium and cultured cells only while the high control containing cells and triton X-100 solution. All evaluated samples were performed in triplicate.

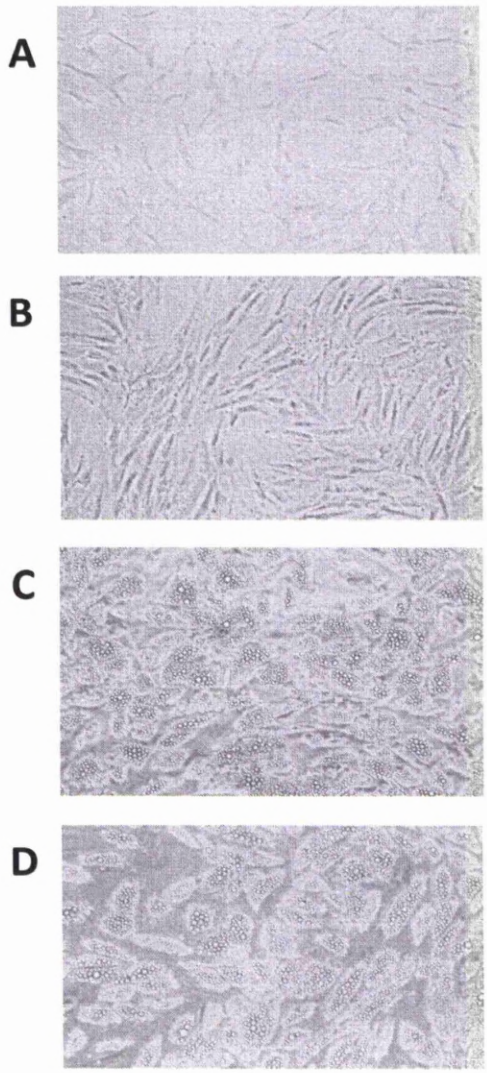

Figure 2.2: Human adipocytes culture system

The photographs above show the differentiation of human preadipocytes *in vitro*, (A) untreated preadipocytes appeared as fibroblast-like cells. (B) preadipocyte at the plating stage. (C) day 12 after the induction period, cells enlarged, attained a polygonal contour and accumulated tiny lipid droplets (D) lipid-filled multilocular cells showing typical morphological features of mature adipocytes *in vitro*.

2.8 Adipocyte cell Culture (SGBS Cell)

2.8.1 Reagents

2.8.1.1 Serum-containing medium

DMEM/Nutrient Mix F12

33 μM Biotin

17 μM Pantothenate

100 units/ml Penicillin

100 μg/ml Streptomycin

10% FCS

2.8.1.2 Feeding Medium

DMEM/Nutrient Mix F12

33 μM Biotin

17 μM Pantothenate

100 units/ml Penicillin

100 μg/ml Streptomycin

10 μg/ml Human Transferrin

10 nM Insulin

100 nM Cortisol

200 pM Triiodothyronine (T3)

2.8.1.3 Induction Medium

DMEM/Nutrient Mix F12

33 μM Biotin

17 μM Pantothenate

100 units/ml Penicillin

100 μg/ml Streptomycin

10 μg/ml Human Transferrin

10 nM Insulin

100 nM Cortisol

200 pM T3

250 nM Dexamethasone

500 mM IBMX

10 mg/ml Rosiglitazone

All media were prepared using a sterilizing filter system in a cell culture fume cupboard. The media prepared was stored in a fridge with a temperature of 4°C. Before use, the media was warmed up to 37°C in a water bath. The culture and feeding media prepared was used within 2 weeks. The induction medium, however, was made fresh immediately before use.

2.8.2 Culture Method

SGBS preadipocytes were kindly supplied by Professor Martin Wabitsch (University of Ulm, Germany). They are a human preadipocyte cell "strain" derived from the stromal-vascular fraction of the subcutaneous white adipose tissue of an infant with Simpson-Golabi-Behmel syndrome. The cell strain found shows a high capacity for adipose differentiation, resulting in mature fat cells which are biochemically and functionally similar to human adipocytes (Wabitsch et al., 2001).

A frozen SGBS cell stock in a cryovial was taken out from the liquid nitrogen and

quickly defrosted in a 37°C water bath. To entirely thaw the cells, 1 ml of pre-warmed culture medium was added to the cryovial and gently pipetted up and down several times. The thawed cell suspension was then transferred to a 25 cm2 flask containing 2 ml of culture medium. The cells were maintained in an incubator at 37°C in a humidified atmosphere with 5% CO2/95% air. The culture medium was changed two to three times a week until 80%-90% of the cells were confluent. At this point, the cells were ready to be passaged and subcultured. Prior to trypsinisation, the cells were briefly washed once with sterilized 1 xPBS. Then, 1 ml of trypsin/EDTA was added to the flask and the flask immediately put in a 37°C incubator for 1 min to help quickly detach the cells. To examine whether the cells were trypsinised completely, the flask was checked under a light microscope. After the cells were entirely detached, 9 ml of culture medium was added in a final volume of 10 ml. The cell suspension was gently pipetted in and out for several times to mix cells evenly. Equal density of preadipocytes was seeded in new flasks, and 6-well or 12-well plates according to the experimental requirement. The culture medium was changed two or three times a week until cells were ready to be induced.

2.8.3 Differentiation of SGBS cells

Differentiation of the cells was initiated 24 h after 100% confluence in the plates. After aspiration of the old medium, the cells were gently washed with pre-warmed 1x PBS three times, and then incubated for 4 days in the FCS-free medium containing 0.25 μM dexamethasone, 500 μM 3-isobutyl-1-methyl-xanthine, 10 nM insulin, 200 pM triiodothyronine (T3), 1 μM cortisol and 2 μM rosiglitazone. Differentiation into adipocytes was examined under a light microscope by phase

contrast at 100x magnification. More than 90% of SGBS cells underwent full differentiation into mature adipocytes after day 10 post-induction.

2.8.4 Harvesting the Cells

The cells were maintained in the feeding medium containing insulin, cortisol and T3, which was renewed two or three times a week. According to the particular experimental design, the cells were collected at several time-points.

2.9 Enzyme-linked immunabsorbant assay (ELISA)

2.9.1 Measurement of IL-6

The total amount of IL-6 in serum or media was measured by enzyme-linked immunosorbent assay (ELISA) (PeproTech, USA) according to the manufacturer's protocol. The stated minimal detectable amount was 50 pg/ml. A 96 well microplate (Fisher .UK) was initially treated with 100 µl capture antibody (100µg/ml) (1:100) diluted in PBS for overnight incubation at room temperature. 300 µl of 1% BSA in PBS was used to block the capture antibody for 1 hour, followed by three washes with 0.05%Tween20 in PBS. 100 µl of serum or plasma was added in duplicate for each subject and incubated for 2 hours at 25°C. The detection antibody 100µl was incubated for 2 hours at 25°C. Detection was initiated with 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)); 150 µl was added to the well. The reaction was stopped by adding 100 µl of 2N H_2SO_4 to each well and absorbance was measured at 405 nm with wavelength correction set at 650 nm. Horseradish peroxidase (HRP) enzymatic activity was responsible for the colour change in the ABTS substrate; there was a relation between the amount of IL-6 and the absorbance at wavelength of 405 nm. Data was collected

and analysed with Microplate Manager V5.2 software (Bio-Rad) to determine the IL-6 concentration in each well.

2.9.2 Measurement of Adiponectin

ELISA measurement of total adiponectin was carried out following the same protocol as for IL-6 using a human adiponectin kit (R&D System). High Molecular weight adiponectin measurement was performed in the department of Clinical Chemistry, University of Liverpool, according to the manufacturer's protocol (Alpco, USA). The sensitivity of the ELISA for total adiponectin and HMW adiponectin was 50 and 0.19 ng/ml respectively.

2.9.3 Measurement of Leptin

ELISA measurement of leptin was carried out following a similar protocol as for IL-6 using human leptin kit (RayBio, UK).

2.10 RNA extraction

The commercially available, Tri-reagent (Sigma) 1 ml was added to the tube and the sample homogenized using either a glass or an electric homogenizer (Polytron Ultra-Turrax T25). The tissue or cells homogenate was transferred into a 2 ml tube and centrifuged at 12,000 g for 10 min at 4°C. The clear supernatant was aspirated and transferred into a fresh 2 ml tube. Care was taken not to aspirate the cell debris pellet or any lipid layer overlying the supernatant.

For RNA extraction, the cells were first collected using Tri-Reagent as described and homogenized by triturating 10 times using a 1 ml syringe with a 23 gauge needle.

After incubating the tissue or cell homogenate at room temperature for 5 min, 200 µl of chloroform was added per ml of Tri-Reagent used. The tube was shaken vigorously for 15 sec by hand and then maintained for 2-3 min at room temperature. This was followed by centrifugation at 12,000 g for 15 min at 4°C, leading to separation of three layers - a colourless upper aqueous phase containing RNA, a white interphase containing DNA and a red phenol phase containing protein. The aqueous layer containing the RNA was transferred into a fresh 2 ml tube, taking care to avoid contamination with either of the other two phases. If protein isolation was required the phenol phase was saved and stored at -20°C.

To minimise any possible genomic DNA contamination, an optional step (recommended by Sigma) was performed. Fifty µl of isopropanol was added to the sample per ml of Tri-Reagent used. This was mixed by vortex, allowed to stand for 5 min at room temperature and then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred to a 1.5 ml tube, taking care not to aspirate in the area where any DNA pellet would have been precipitated.

Isopropanol was then added to the sample (450 µl per ml of Tri-Reagent used in sample preparation), followed by vortexing, incubation of the sample for 10 min at room temperature and centrifugation at 12,000 g for 10 min at 4°C. After these steps the precipitated RNA pellet could usually be identified at the base of the tube.

The supernatant was discarded and 700 µl of 75% ethanol added to the RNA pellet. This was followed by vortexing and centrifugation at 10,000 g for 10

min at 4°C. The supernatant was again poured off, and any remaining volume aspirated by pipette. The RNA pellet was allowed to air dry for 2-3 min and then dissolved by adding 10-15 µl of ultra-pure water. Samples could be stored temporarily if required at -20°C prior to DNase treatment and/or quantification. Scissors, tweezers and glass homogenizers or electric homogenizer blade components were soaked in 1% Virkon solution for 1-2 h for decontamination, then cleaned of any tissue debris prior to rinsing.

2.10.1 Quantification of RNA

The RNA extracted samples were passed through a spectrophotometer (BioPhotometer) to determine their concentration. RNA was diluted in RNase-free water to give a 1 in 70 dilution. The RNA tubes were measured immediately making sure that the cuvette was rinsed with water before each use. The RNA concentration was determined from the absorbance at 260 nM (Nielsen, 2011) . To determine the concentration of RNA, the Beer-Lambert law was used.

[RNA] µg/µl = A260 x 44 x 70 / 1000

44= coefficient number for single stranded RNA.

2.10.2 Integrity

2.10.2.1 Reagents

Ultra-pure water

Sample buffer (1 ml 40% glycerol, 40 µl saturated BPB

solution) Reagents for agarose gel electrophoresis

RT-PCR was performed using the isolated RNA samples. Once the cDNAs had

been synthesized, their quality was checked by performing PCR using primers for the housekeeping gene β-actin and running the products on a gel. The band intensity indicated the quality of the cDNA sample from which the integrity of the original RNA template could be inferred. Faint or undetectable bands suggested degradation of the isolated RNA or a failed RT reaction. The former could be investigated by checking the RNA integrity directly and if found to be significantly degraded, the RNA was re-extracted from the tissue.

mRNA comprises 1-5% of a total RNA sample, whereas ribosomal RNA (rRNA) makes up >80% of total RNA, the majority of that consisting of 28S and 18S rRNA species (in mammals). The method used to directly check RNA integrity (denaturing gel electrophoresis) relies on the assumption that rRNA quality and quantity reflect that of the underlying mRNA population, with the caveat that there may be a degree of difference between the integrity of the long lived and abundant rRNA molecules and that of the underlying mRNA population, which turns over much more rapidly (Palmer & Prediger, 2005).

2.10.2.2 Method

One µg of RNA was diluted with RNase-free water to a total volume of 5 µl in a 0.2 ml tube and 2 µl of sample buffer added. The sample was mixed by vortexing briefly, loaded onto a 1 % agarose gel and run at 80-100 V for 30-40 min. An image of the gel was recorded digitally. An intact RNA sample would have two clearly visible and well- defined bands representing 28S and 18S rRNA with little or no visible debris at the base of the gel. RNA degradation would be inferred from smudging or absence of the rRNA bands and a strong signal at the base of the gel.

2.11 Reverse transcriptase –polymerase chain reaction (RT-PCR)

2.11.1 RT-PCR

2.11.1.1 Reagents

iScript cDNA Synthesis Kit:

5X cDNA synthesis kit buffer

iScript enzyme mixture

Nuclease-free water

2.11.1.2 Method

0.5 µg RNA was reverse transcribed with iScript™ cDNA Synthesis Kit (BioRad) in the presence of mixture of oligo (dT) and random hexamer primers in a total volume of 10 µl. The samples were heated at 25°C for 5 min to remove secondary structures in the mRNA. Each sample was reverse transcribed to cDNA at 42°C for 30 min. The samples were further heated at 85°C to inactivate the iScript Reverse Transcriptase. The cDNA samples were stored at -20°C until required.

2.11.2 PCR

The PCR generates multiple copies of a cDNA. A master mix was prepared that includes the DNA, which was placed in a PCR thermal cycler. Firstly, the temperature was increased to 94°C, which causes the DNA double helix to separate (denaturation). Secondly, the temperature was lowered, which causes the primers to anneal (attach) to the double stranded DNA. Primers are specific DNA oligonucleotides that match to specific DNA sequences, which initiate the process of making new DNA. Thirdly, an enzyme called Taq polymerase (ready

mix) was used to make new DNA chains. The temperature was again increased and the enzymes made new DNA to match the DNA strands that were separated. The cycles were repeated many times; after only 20 cycles, there would be a million copies of the original DNA.

2.11.2.1 Reagents

DNase/RNase free water

10 µM each sense and antisense primer mix

ReddyMix PCR Master Mix Kit:

1.1xReddyMix

2.11.2.2 Method

This procedure was carried out using sterile 0.2 ml RNA/DNase-free tubes and filter tips and reactions were performed using a thermal cycler (PCR Express). A master mix containing 11.25 µl of 1.1x ReddyMix, 0.25 µl of primer mix and 0.5 µl of (ultra- pure) water per sample was prepared, vortexed and centrifuged. Twelve µl of this master mix were transferred into each tube and 0.5 µl of the appropriate cDNA added for a 12.5 µl final reaction volume (all volumes proportionately scaled up for a 50 µl reaction); ultra-pure water was used for the 'no template' control. The tubes were vortexed, centrifuged and placed into the cycler with the PCR program set as follows:

94°C, 2 min (denaturation)

20-38 cycles (specified cycle number for each primer pair) of:

i. 94°C, 20 sec (denaturation)

ii. 54°C, 30 sec (annealing; T_a)

iii. 72°C, 30 sec (extension)

iv. 72°C , 5 min (final extension)

2.11.3 Agarose electrophoresis

2.11.3.1 Reagents

Agarose

5 mg/ml ethidium bromide

10x TBE buffer

100 bp DNA ladder (GeneRuler or ReddyRun Super-ladder low 100 bp)

Distilled water

The gel casting tray was prepared by sealing the ends with autoclave tape. The agarose was weighed directly into a 500 ml conical flask, the 1x TBE added and the flask heated for ~2 min in a microwave (flask taken out and swirled every 30 sec) until the agarose was completely dissolved. The solution was cooled to approximately 50° C prior to adding the ethidium bromide. The mixture was poured into the gel casting tray avoiding any bubble formation. The combs were then placed into their slots and the gel allowed to set for at least 15 min. The autoclave tape was removed and the tray with the gel placed in the gel tank. The tank was filled with 1 x TBE until the gel was submerged in the buffer before removing the combs.

For analyzing the PCR products on the gel, 10 µl of each sample was pipetted into separate wells. Two µl of DNA ladder (Helena BioSciences, UK) was loaded

near the samples as a reference to assess whether the PCR product was located at the expected size. The end wells were avoided if possible, as these are most likely to run aberrantly. The gel was run at 85-100 V for 30-60 min or until the samples had run an approximately similar distance. For band detection, the gel was placed on a UV transilluminator (2011 Macrovue). The images were recorded using a DC120 digital camera and analysed using ID 1 image analysis software.

2.12 Real time–polymerase chain reaction

Real-time PCR can be used for absolute or relative quantitation of the initial amount of template in a sensitive, specific and reproducible manner. It is preferable to alternative forms of PCR quantification because it quantifies the initial template cDNA rather than the final amount of amplified PCR product. This is possible as real- time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle. The fluorescent signal increases in direct proportion to the amount of PCR product. During the exponential phase of the PCR reaction, there is a significant increase in the amount of PCR product. By monitoring the fluorescence, the cycle at which this phase commences can be determined. The more initial template there is, the earlier the fluorescent signal will be significantly increased.

Real-time PCR also offers a wide dynamic range of up to 10^7 -fold, compared to only 10^3 -fold in conventional PCR, which means that large variations in template amount can be quantified sensitively and accurately.

2.12.1 Taqman Q-PCR

The real-time PCR work performed in this study employed the Taqman system

(Applied Biosystems), which involves the use of a primer pair and a Taqman probe. The probe is an oligonucleotide with a fluorescent (reporter) dye (usually FAM) on the 5' base, and a quenching dye (usually TAMRA) typically on the 3' base. When the probe is intact, energy is transferred from the reporter dye to the nearby quenching dye molecule (FRET - fluorescence resonance energy transfer) rather than emitted as fluorescence (Giulietti et al., 2001). During the annealing phase of the PCR cycle, the probe binds to an internal region of a PCR product and when the Taq polymerase replicates a template onto which a probe is bound, its 5' exonuclease activity cleaves the probe. This separates the reporter and quenching dyes and ends the FRET. The reporter dye now starts to emit fluorescence (at 518 nm for FAM) which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence (ΔR) of the reporter dye (usually plotted as ΔR_N after normalisation of the reporter dye signal to a reference dye [usually ROX] to correct for differences in reaction mix volumes between wells) (Giulietti *et al.*, 2001).

2.12.2 Primers and Taqman probes

Primer and Taqman probe sequences are detailed in Table 2.1, together with the optimized final reaction concentrations. For a new primer/probe set, optimal primer concentrations were determined by performing an assay with final concentrations of sense and antisense primers ranging from 50 nM to 900 nM, in all possible combinations, with the probe concentration fixed at 200 nM. The resulting amplification plots were then examined and the combination with the lowest C_T and, the highest ΔR_N was selected. Taqman probe optimisation was

then performed at concentrations from 25-225 nM using the optimised primer concentrations and the amplification plots were used to select the optimal probe concentration.

Gene	Sequence (5'–3')	Size, bp
Adiponectin		73
Forward	CCCAAAGAGGAGAGAGGAAGCT	
Reverse	GCCAGAGCAATGAGATGCAA	
Probe	TTCCCAGATGCCCCAGCAAGTGTAAC	
Leptin		93
Forward	CCAAAACCCTCATCAAGACAATT	
Reverse	AGTCCAAACCGCTGTCTTTCTG	
Probe	TGACATTTCACACACGCAGTCAGTCTCCT	
IL-6		83
Forward	GGTACATCCTCGACGGCATCT	
Reverse	GTGCCTCTTTGCTGCTTTCAC	
Probe	TGTTACTCTTGTTACATGTCTCCTTTCTCAGGG	
MCP-1		104
Forward	CATAGCAGCCACCTTCATTCC	
Reverse	TCTGCACTGAGATCTTCCTATTGG	
Probe	CAGCCAGATGCAATCAATGCCCC	
POLR2A		81
Forward	ATGGAGATCCCCACCAATATCC	
Reverse	CATGGGACTGGGTGCTGAAC	
Probe	TGCTGGACCCACCGGCATGTTC	
LPL	Assay ID Details(Applied Biosystem):Hs 00173425_ml	
HSL	Assay ID Details(Applied Biosystem):Hs 00181192_ml	
LDL-R	Assay ID Details(Applied Biosystem):Hs 00181192_ml	
C3	Assay ID Details(Applied Biosystem):Hs 00163811_ml	

Table 2.1 Real-time PCR primer and Taqman probe sequences

The table shows the sequences of the primers and Taqman probes employed in real- time (q) PCR analysis for (relative) quantitation of gene expression.

2.12.3 Preparation of 96 well plates for real-time PCR

2.12.3.1 Reagents

qPCR Core Kit:

10x reaction buffer

50 mM magnesium chloride

5 mM dNTP mix

5 U/µl Hot Goldstar enzyme

Sense primer, antisense primer and Taqman probe stock solutions

Ultra-pure water

2.12.3.2 Method

Working solutions of the primers and probes were prepared at 10x concentration (e.g.9000 nM for a final reaction concentration of 900 nM) by diluting the stock solution with ultra-pure water. A master mix was made up which contained all the necessary components for the PCR, minus the cDNA template as follows:

10 x reaction buffer	2.5 µl per well (1x final concentration)
50 mM MgCl	2.5 µl (5 mM)
5 mM dNTP	1 µl (200 mM)
Forward primer	2.5 µl
Reverse primer	2.5 µl
Taqman probe	2.5 µl
5 U/µl Hot Goldstar enzyme	0.125 µl (0.025 U/µl)
Ultra-pure water	11.375 µl

Once the master mix had been made up, 25 µl were aliquoted to each well. One

µl of the appropriate template cDNA was then added to each well for a total reaction volume of 26 µl and the plate sealed with an optical cover. All samples were run in duplicate, or triplicate if practical. No primer and no template controls were included on the plate for each set of primers and probe. The plate was spun at 1000 rpm for 20 sec to ensure the reaction mix was not sticking to the well sides. If the assay was not to be performed immediately, the plate could be stored for up to 5 days at 4°C. If a set of samples (comprising several groups) required analysis over several plates, at least one group (usually the control group) was present on all plates. Data were normalized such that the mean ΔC_T for the control group had the same value for each assay, enabling the data on the different plates to be directly compared.

Reactions were performed using an Mx3005P cycler (Stratagene). Default conditions were used except that the reaction volume was specified as 26 µl. Once the cover temperature had reached 105°C, the plate was inserted into the heat block and an optical pad placed on top so that the holes aligned with the well positions. The cover was screwed into position and the run started.

Amplifications were performed commencing with a 2 min activation stage at 50°C, then a 10 min denaturation stage followed by 40 cycles consisting of a denaturation step of 15 sec at 95°C and a combined primer annealing and extension step for 60 sec at 60°C. Data were collected automatically by the software, and analysed once the run was complete (total run time was ~2 h).

2.12.3.4 Analysis of real-time PCR

The amplification plots were displayed in log scale using the Sequence Detector

software and the threshold manually adjusted to ensure that it was clear of any noise. The software automatically recalculated the C_T value for each well (C_T defined as the cycle number at which the fluorescence emitted from the well crossed the threshold). The results were exported as a Microsoft Excel file and gene expression analysed by relative quantitation with the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). All samples were normalised to values of β -actin, the difference of C_T values between the control and treated samples were calculated and the relative fold changes obtained between the two groups. The formulae used for calculation are as follows:

ΔC_T= C_T (Target gene) - C_T (Reference gene)

$\Delta\Delta C_T$ = Mean ΔC_T (patient samples) - Mean ΔC_T (control samples) Fold Change=$2^{-\Delta\Delta CT}$

2.13 Statistics

Data are expressed as mean ± SEM or mean ± SD as appropriate. The statistical significance of the difference between the means of two groups of samples was assessed by Student's *t*-test. For comparison between groups, ANOVA was used followed by *post hoc* testing with Bonferroni correction. Paired Student test was used to determine the significance between the pre and post dialysis data means. Differences between study groups in tissue staining score in chapter 6 were tested using Mann U test. Differences were considered to be significant when the *p* value was less than 0.05 ($p < 0.05$).

CHAPTER 3

LIPOPROTEIN SUBFRACTION PROFILE OF PATIENTS WITH END STAGE RENAL DISEASE

3.1 Introduction

End-stage renal disease (ESRD) is a major public health problem worldwide. Although dialysis is an effective therapy, ESRD is associated with other major health problems. Dialysis patients have been noted to have shorter life expectancy compared to the general population. The most common cause of death in ESRD is cardiovascular disease (CVD) (Wheeler, 2007). CVD mortality in younger ESRD patients is about 500-fold higher than healthy patients and was 5-fold higher in older patients (Sarnak, 2003). This strong association is not fully understood. However, many risk factors have been proposed to contribute to CVD in ESRD (section 1.3 and 1.4). These factors become worse over time in keeping with ESRD progression (Fellstrom et al., 2009). Several studies pointed out that risk factors in ESRD might have different effects from that observed in general population (Yamamoto and Kon, 2009).

Dyslipidaemia is a well-known risk factor of CVD in ESRD (Anavekar and Pfeffer, 2004, Rucker and Tonelli, 2009). The characteristic dyslipidaemia of ESRD is not consistent between patients. Dyslipidaemia in ESRD depends upon many factors including the degree of renal failure, etiology of primary renal disease, type of dialysis and the presence of proteinuria (Fellstrom et al., 2009, Yamamoto and Kon, 2009). ESRD-induced dyslipidaemia is characterized by hypertriglyceridaemia, low high-density lipoprotein (HDL) and normal or near normal levels of plasma cholesterol (Attman and Samuelsson, 2009). However, these characteristics are prone to modification depending on the type of dialysis, co-morbid disease and medications (Chan et al., 2008). Hypertriglyceridaemia is regarded as a common feature in kidney disease (Yamamoto and Kon, 2009). In

addition to lipid concentration changes in ESRD, lipoproteins also undergo an alteration in composition (Lee et al., 2002).

The purpose of the studies in this chapter was to characterise the distribution of dyslipidaemia in relation to ESRD and its treatment by dialysis. The specific aim was to determine the lipoprotein subfraction pattern and to explore the altered lipoprotein composition. A further objective was to investigate whether these abnormalities are found both in haemodialysis and peritoneal dialysis.

3.2 Methods

3.2.1 Subjects

See section 2.2.1

3.2.2 Blood

See section 2.2.4

3.2.3 Biochemical analysis

Urea, creatinine, cholesterol, TGs, LDH and total protein concentrations were measured on a Roche Modular™ SWA analyser using colorimetric or ensymatic assays following the procedures provided by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

3.2.4 Lipoprotein fractionation

Lipoprotein fractions were done using a self-generating iodixanol gradient. 1.2 ml of 60% iodixanol was added to 4.8 ml of EDTA/plasma and mixed. 4.8 ml of 6% (w/v) iodixanol in phosphate buffer saline was placed in a 13x35 mm polyallomer

centrifuge tube. The iodixanol-plasma was carefully layered underneath the 6% (w/v) iodixanol solution. Approximately 1 ml of PBS was then added to the top of each tube and the tubes centrifuged at 65,000 rpm and 16 °C for 190 min (VTI 65.1 Rotor, Beckman L8-80 ultracentrifuge). 1 ml aliquots were removal from the top first by automated fractionators (Autodensei-flow 451-7200, Labconco) into 1.5 ml microfuge tubes. The aliquots were stored at -70 °C until further analysis.

3.2.5 Statistics

Data are expressed as mean ± SEM or mean ± SD as appropriate. For comparison between groups, ANOVA was used followed by *post hoc* testing with Bonferroni correction. Paired Student test was used to determine the significance between the pre and post dialysis data means. Differences were considered to be significant when the p value was less than 0.05 ($p < 0.05$).

3.3 Results

3.3.1 Clinical and biochemical characteristics

Clinical and biochemical data of all subjects are reported in Table 3.1. Altogether, 18 ESRD patients and 9 control subjects (healthy) completed the study. There was no difference in age and body mass index (BMI) in the three groups. The mean creatinine concentration was 938.8 (±159.4) μmol/L in the haemodialysis patients, 775 (± 363.3) μmol/L in the peritoneal dialysis group and 87.6 (± 31.8) μmol/L in the control groups. The mean urea concentration was 23.8 (± 4.9) mmol/L in the haemodialysis patients, 21.5 (±5.8) mmol/L in the peritoneal dialysis group and 5.7 (± 2.3) mmol/L in the control groups. Post-dialysis mean of creatinine was

401.9 (±128.3) μmol/L and for urea 8.5 (± 4.1) mmol/L. Higher creatinine and urea levels confirmed the presence of renal failure in both the HD and PD groups.

3.3.2 Plasma lipid profile

Initially, lipid parameters (Cholesterol, Triglycerides, LDL-cholesterol and HDL-cholesterol) were measured for all subjects. As shown in table 3.2, haemodialysis patient groups (pre and post) had increased plasma triglyceride and decreased HDL cholesterol levels. There was no difference in LDL-cholesterol compared with controls. Interestingly, peritoneal dialysis patients exhibited decreased in TG compared with the Pre-HD and Post-HD ($p < 0.05$) and increased in HDL-cholesterol compared with the Pre-HD ($p < 0.01$). Peritoneal dialysis patients exhibited no significant changes in plasma lipids profile compared the controls.

All patient groups had normal or near normal serum cholesterol and LDL cholesterol levels.

	H	HD	PD
Number	9	11	7
% Male	100%	100%	100%
Ethnicity	9 Caucasian	11 Caucasian	7 Caucasian
Mean age (years)	45.1± 7.9	47.5 ± 14	45.4 ± 8.1
BMI (Kg/m^2)	26.7 ± 2.9	29.2 ± 5.5	27.6 ± 3.8
Smoker	2	2	2
Diabetes	0	0	1
CVD events	0	5	1
Hypertension	0	10	7
Medication			
% Statin	0%	36.4%	71.4%
Miscellaneous *			

Table 3.1: Clinical and biochemical characteristics of subjects

H, Healthy group; HD, Haemodialysis group; PD, Peritoneal dialysis group; BMI, body mass index. *Please see appendix 2 for full list of medication

Data are means ± SD

	H	Pre-HD	Post-HD	PD	Significance by ANOVA
TC (mmol/L)	4.7 ± 1.1	3.9 ± 1.2	4.4 ± 1.4	4.5 ± 1.2	NS
TG (mmol/L)	1.4 ± 0.6	2.9 ± 1.5	3.0 ± 1.6 [b]	1.3 ± 0.6 [d]	$P < 0.001$
HDL-C (mmol/L)	1.3 ± 0.3	0.8 ± 0.2 [a]	0.9 ± 0.2 [b]	1.3 ± 0.3 [c]	$P < 0.0001$
LDL-C (mmol/L)	2.8 ± 1.2	1.8 ± 1.0	2.1 ± 1.3	2.6 ± 1.1	NS

Table 3.2 Plasma lipid profile of subjects

Data are mean ± SD

Abbreviations: H, healthy group; Pre-HD, pre-haemodialysis; Post-HD, post-haemodialysis; PD, peritoneal dialysis; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

Differences among the four groups were analysed by one-way ANOVA. [a,b,c,d] Significant difference between H and Pre-HD (a), between H and Post-HD (b), between Pre-HD and PD (c) and between PD and Post-HD (d) assessed by multiple comparison (Bonferroni correction, $p < 0.05$).

3.3.3 Effects of HD on biochemical parameters

To investigate the effect of dialysis on the lipid profile, pre and post dialysis samples were compared. Plasma concentrations of cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol were determined before and after haemodialysis. Cholesterol and HDL-cholesterol concentrations were significantly increased after haemodialysis as shown in Figure 3.1 ($p < 0.001$ and 0.0001 respectively). No significant change was observed in triglycerides and LDL-cholesterol concentrations.

3.3.4 Cholesterol distribution of lipoprotein subfractions

We determined the distribution of the conventional VLDL, LDL, and HDL isolated by the iodixanol density gradient in control (Fig. 3.2), HD (Fig. 3.3), and PD groups (Fig. 3.4) .The lipoproteins show distinct distributions in the gradient. VLDL was recovered in fractions 1–2 (density 1.007); LDL in fractions 4–7 (density 1.016– 1.043); and HDL in fractions 8–9 (density 1.043–1.076). Figure 3.5 shows the differences between these groups. Cholesterol distribution of lipoprotein fractions showed that a significant increased VLDL ($p < 0.05$) and presence of small dense LDL in patients groups compared to control subjects. Peritoneal dialysis patients had increased and presence of small dense LDL and a shift in cholesterol to IDL. Multiple comparison analysis (Table 3.3) showed a significant increase in fraction 1 in Pre-HD compared to healthy groups ($p < 0.05$) as well as an increase in fraction 10 in PD compared to healthy groups ($p < 0.05$).

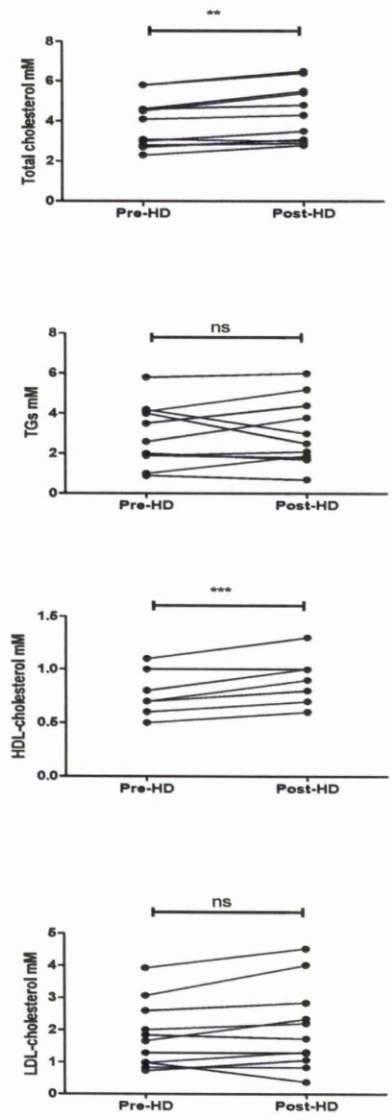

Figure 3.1 Plasma lipid profile before and after HD

Blood samples were collected immediately before and after haemodialysis. Total cholesterol (A), Triglycerides (B), LDL-cholesterol (C) and HDL-cholesterol (D) were investigated. Values are mean ± SD. Change in cholesterol was positive in 9 subjesta, TG in 6 subjects, LDL-C in 8 subjects and HDL-C in 10 subjects.
*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significant difference from the Pre-HD group (n=11)

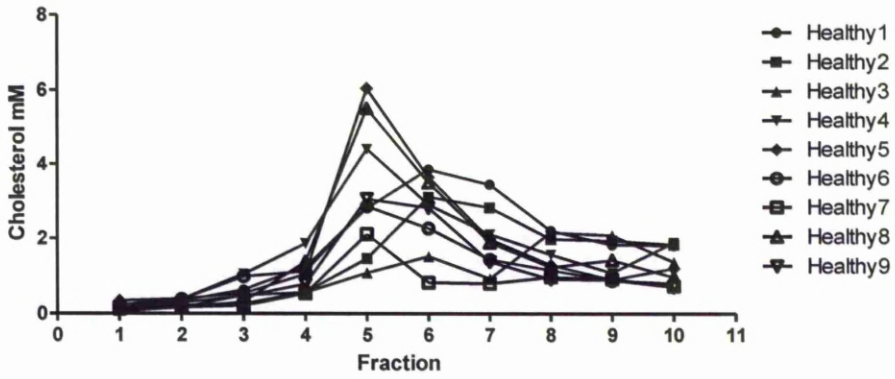

Figure 3.2: Density profile of the iodixanol gradient and cholesterol distribution of plasma lipoproteins (control group)

Separation of plasma lipoproteins on iodixanol gradients using Optiseal TM tubes in the VTi 65.1 rotor. Fractions (1 ml) were taken from the top of the gradient. 10 fractions were analysed for total cholesterol and total protein in order to identify the Lipoproteins fraction. There was similarity in VLDL fractions (1-2) and HDL (8-9) while LDL fractions (4-7) showed a peak variation between the subjects.

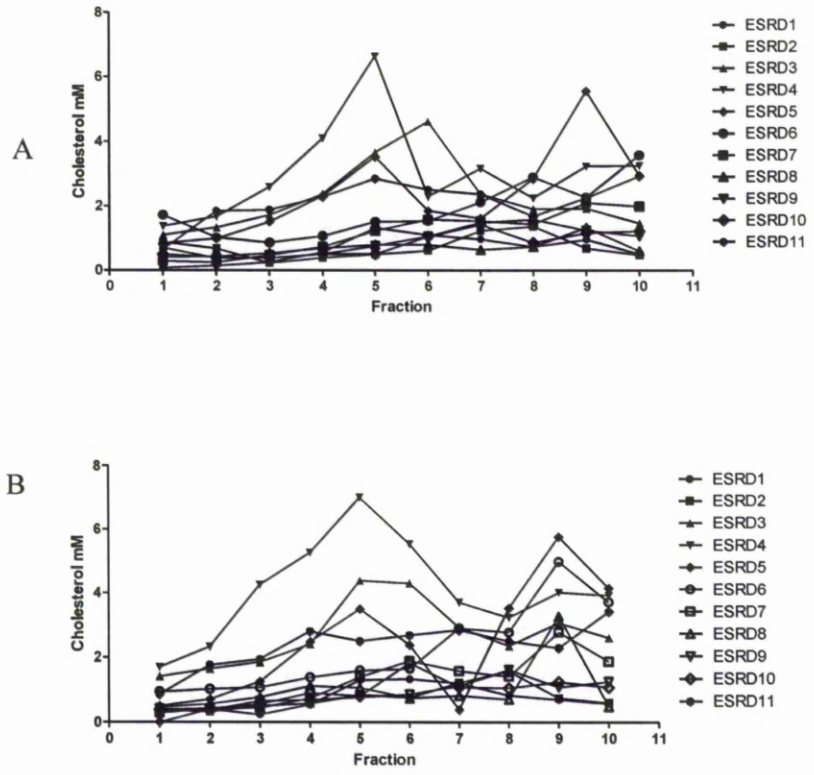

Figure 3.3: Density profile of the iodixanol gradient and cholesterol distribution of plasma lipoproteins (haemodialysis group)

(A) pre-dialysis and (B) post-dialysis groups.

Separation of plasma lipoproteins on iodixanol gradients using Optiseal TM tubes in the VTi 65.1 rotor. Fractions (1 ml) were taken from the top of the gradient. 10 fractions were analysed for total cholesterol and total protein in order to identify the lipoproteins fractions. In contrast to the healthy group, dialysis patients showed a big variation in all fractions with a markedly different pattern in some patients.

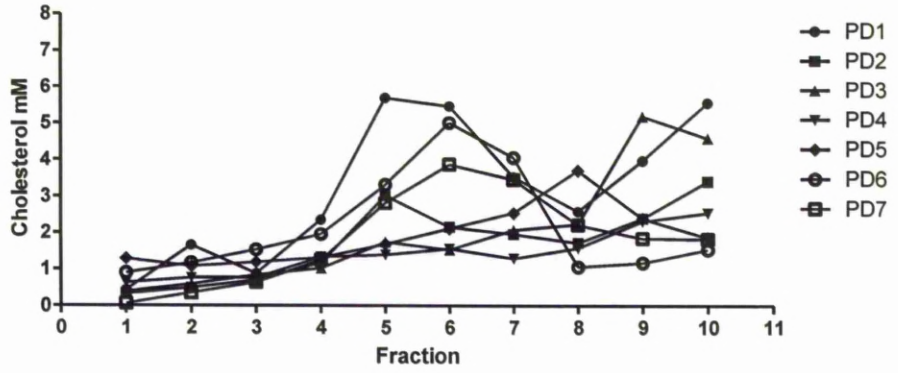

Figure 3.4: Density profile of the iodixanol gradient and cholesterol distribution of plasma lipoproteins (peritoneal group)

Separation of plasma lipoproteins on iodixanol gradients using Optiseal TM tubes in the VTi 65.1 rotor. Fractions (1 ml) were taken from the top of the gradient. 10 fractions were analysed for total cholesterol and total protein in order to identify the lipoproteins fractions. PD group showed similarity in VLDL fraction and a distinct variation in LDL and HDL fractions

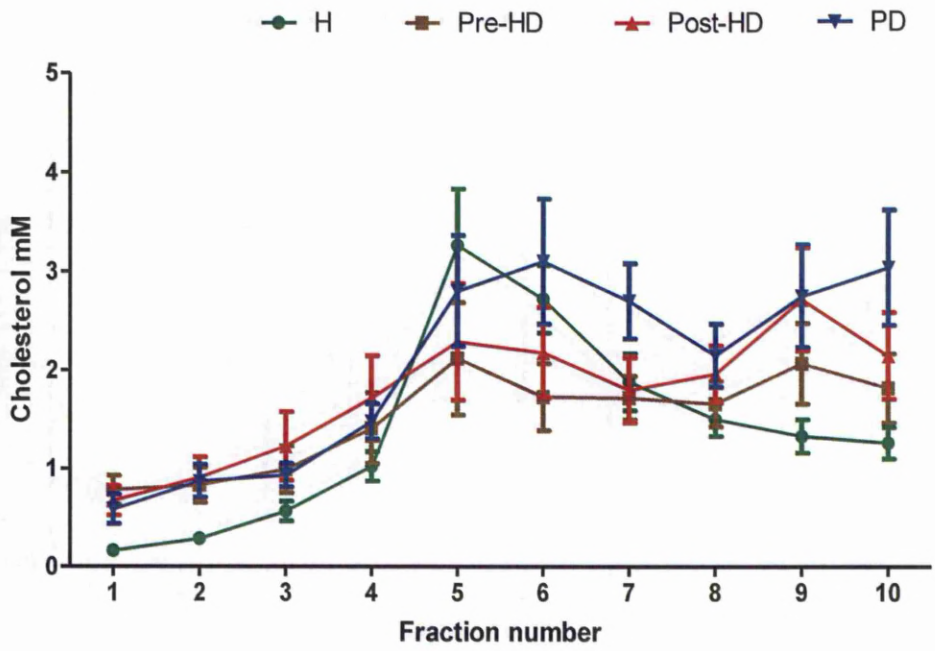

Figure 3.5: Density profile of the iodixanol gradient and cholesterol distribution of plasma lipoproteins (Pooled Data)

Means of Iodixanol lipoprotein cholesterol distribution in all groups; control (*green line*), pre-haemodialysis group (*brown line*), post-haemodialysis group (*red line*) and peritoneal dialysis group (*blue line*). *p* value shown in table 3.3. Values are means ± SEM ($n = 9$ for control group, $n = 11$ for HD group and $n = 7$ for PD group).

Fraction		Significance by ANOVA
1	VLDL	$P < 0.05$ [a]
2		$P < 0.0518$
3		NS
4	LDL	NS
5		NS
6		NS
7		NS
8	HDL	NS
9		NS
10		$P < 0.05$ [b]

Table 3.3: Multiple comparison analysis for lipoprotein fractions

HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very density lipoprotein.

Differences among the four groups were analysed by one-way ANOVA. [a, b] Significant difference between healthy and pre-haemodialysis group (a), between healthy and peritoneal group (b) assessed by multiple comparison (Bonferroni correction, $p < 0.05$). ($n = 9$ for control group, $n = 11$ for HD group and $n = 7$ for PD group. NS, not significant.

3.4 Discussion

ESRD patients have an extremely high rate of morbidity and mortality from CVD. Although many factors other than dyslipidaemia may contribute to the CVD in ESRD, dyslipidaemia plays a role both in the progression of ESRD and in generating CVD events. Dialysis patients in this study had dyslipidaemia with many different patterns. Haemodialysis patients had increased serum triglycerides and decreased HDL-cholesterol compared to the control subjects. Hypertriglyceridaemia and low HDL-cholesterol are considered as the main features of dyslipidaemia in ESRD. The exact mechanism for hypertriglyceridaemia in ESRD is not fully understood and seems to be multifactorial (Prinsen et al., 2003).

Hypertriglyceridaemia can result from the accumulation of intact and/or partially metabolised TG-rich apo B containing lipoproteins. This accumulation, in turn, is related to defects in lipolytic enzyme activity in ESRD, especially lipoprotein lipase (LPL) and hepatic lipase. LPL plays a major role in regulation of plasma lipoprotein and energy metabolism.

LPL deficiency leads to impaired VLDL and chylomicron metabolism. Several studies have reported a decrease of post-heparin activity in ESRD (Vaziri, 2009). Moreover, LPL down-regulation has been demonstrated to be associated with hypertriglyceridaemia in ESRD (Vaziri and Liang, 1996). Decreased activity of LPL in ESRD is attributed to the effect of frequent heparin (during dialysis treatments) that diminishes the pool of endothelial-bound lipoprotein lipase (Vaziri, 2009). LPL is activated by apoC-II and inhibited by apo C-III. Apo C-

II/Apo C-III ratio has been reported to be low in ESRD patients (Alsayed and Rebourcet, 1991). However, it has been reported that LPL may not be the main cause for impaired degradation of TG-rich apo B lipoproteins in ESRD (Lee et al., 2002). Lipoproteins may be abnormally altered leading to decreased ability of enzymes to hydrolyze these particles. This was noted, for example, with isolated VLDL from haemodialysis patients which acted as a poor substrate for bovine lipoprotein lipase *in vitro* (Arnadottir et al., 1996). Moreover, hypertriglyceridaemia can be explained by an overproduction of TG-rich apo B-containing lipoproteins (Prinsen et al., 2003).

Consistent with the previous finding, cholesterol plasma levels were within or below normal values with no change seen in the gene or protein expression of enzymes involved in cholesterol metabolism such as HMG-CoA reductase (Pandak et al., 1994) or in hepatic LDL receptor (Liang and Vaziri, 1997b).

Heparin was given as a 1000 IU loading dose and 1000 IU every hour for four hours totaling 5000 IU over 4 hours. This is a small dose over the 4 hour period which appears to have had a negligible effect on circulating lipoprotein metabolism since pre and post HD triglycerides were similar. It is therefore unlikely that heparin had a major effect on circulating lipids and composition of lipoproteins following HD.

The results demonstrated a marked reduction in HDL-cholesterol in the haemodialysis group. Low HDL-cholesterol has been reported as a risk factor for atherosclerosis and cardiovascular disease in the general population (Expert Panel on Detection and Treatment of High Blood Cholesterol in, 2001, Pekkanen et al.,

1990) . In ESRD patients, low HDL-C was also associated with coronary artery disease (Koch et al., 1997). It has been reported that in some ethnic groups, plasma HDL-cholesterol levels are increased after haemodialysis and peritoneal dialysis (Burrell et al., 1991). All recruited subjects in this study were Caucasian. According to National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (NCEP-ATP III), lipid management can be applicable to all population (Expert Panel on Detection and Treatment of High Blood Cholesterol in, 2001). However, African-Americans showed higher mortality from CVD compared with whites (Sumner et al., 2005). LDL-cholesterol levels are similar in white and black races where HDL-cholesterol levels are higher in blacks (Vega et al., 1998). Lp(a) levels were found to be significant higher in blacks compared to whites (Emerging Risk Factors et al., 2009).

Although many studies have reported that peritoneal dialysis is associated more with dyslipidaemia rather than haemodialysis (Attman et al., 2003), data obtained in this study was unable to detect any significant alterations in lipid profile. However, the failure to detect a change in lipid profile could be explained by the low number of recruited subjects in this group and also that all patients were unavoidably receiving lipid lowering medications, such as statins.

To further explore uraemia-induced dyslipidaemia, lipoproteins were separated using iodixanol gradient ultracentrifugation and the present studies, for the very first time using this technique, clarifies and quantifies discrete lipoprotein fractions in ESRD.

The findings were consistent with published work in this field. Results showed that both hamodialysis and peritoneal dialysis groups had increased levels of VLDL. VLDL is primarily produced by the liver. VLDL in ESRD has a reduced catabolism in ESRD due to the low activity of lipolytic enzymes or the change in composition or from the increased content of apo C-III (Attman et al., 1999). The lipoprotein separation pattern in the present study also indicated the peritoneal dialysis group had more atherogenic lipoproteins, namely IDL and small dense LDL. It has been speculated that these lipoproteins are associated with atherosclerosis in ESRD (Shoji et al., 2001). Moreover, IDL was considered as an independent risk factor for aortic atherosclerosis in ESRD patients (Shoji et al., 1998). It has been suggested that long-term exposure to glucose presented in the peritoneal dialysate, may affect the glycaemia and therefore the lipid profile in the PD group. However, in a very recent study (Li and Fengxian, 2011) have reported that glucose does not contribute to the abnormal lipid profile in peritoneal dialysis. IDL is produced as a result of the VLDL metabolism and removed by the liver via LDL-receptor related protein (LPR). Moreover, it has been reported that LRP is downregulated in ESRD leading to high levels of the atherogenic remnant lipoproteins (Kim and Vaziri, 2005).

Although some reports have suggested that statin treatment may be associated with an increase in HDL-cholesterol (Kostapanos et al., 2009), these changes are generally considered less important than the LDL lowering effect of statins. In this study, HDL-cholesterol distribution was increased in both haemodialysis and peritoneal dialysis. The studies in thesis showed that HDL was smaller following PD compared with healthy group.

Although presence of dyslipidaemia is well established in ESRD patients, treatment of dyslipidaemia indicates that using lipid lowering drugs have a limited effect. Many studies have been shown that not all ESRD groups achieved therapeutic targets (Marrs and Saseen, 2010). Studies that examined the potential benefits of statin in the light of CVD risk associated with haemodialysis failed to show an improvement in outcome. The 4D (Die Deutsch Diabetes Dialysis) study showed no significant reduction of CVD by using atorvastatin in diabetic ESRD patient when used for around 2 years (Wanner et al., 2004). A study to evaluate rosuvastatin in subjects on regular haemodialysis, AURORA, recruited 2775 individuals on haemodialysis but showed no significant reduction of the CVD risk associated with ESRD (Fellstrom et al., 2009). However, a recent study employing cholesterol absorption inhibitor Ezetimibe showed a reduction in CVD outcomes (Baigent et al., 2011). Therefore the importance of LDL cholesterol reduction in ESRD is still unclear.

A number of limitations in this study were noted. Firstly, relatively low numbers of patients were studied, since the main goal was detailed metabolic investigation. Furthermore, we present data on lipoprotein subfraction cholesterol distribution in non-fasting samples collected before initiation of the dialysis and after. This may have affected the lipoprotein distribution and lipids profile observed in the present investigations. However, a number of studies have reported that non-fasting lipid measurement has no significant effect in the general population (Langsted and Nordestgaard, 2011), ESRD patients (Nishizawa et al., 2003) and the diabetic population (Lund et al., 2011). Additionally, other analytes were not measured in the lipoprotein subfractions such as triglycerides, and these may differ in ESRD

and represent future directions for investigation. Finally, the data presented in this study are cross-sectional and clear causation cannot be determined.

In conclusion, an atherogenic lipoprotein profile is seen in ESRD patients. These findings suggest that differences in lipoprotein cholesterol distribution exist in ESRD and have the potential to contribute to CVD in ESRD. Overall, these data reflect the need for further investigation of the contribution of lipoprotein to CVD risk in patients with ESRD.

CHAPTER 4

EFFECT OF URAEMIC LIPOPROTEINS ON THE LIPID METABOLISM RELATED GENES EXPRESSION IN HUMAN ADIPOCYTES *IN VITRO*

4.1 Introduction

Lipoproteins are considered essential players in lipid metabolism and include LDL, HDL, VLDL and chylomicrons. Dysregulation of lipoprotein metabolism may contribute to the pathogenesis of atherosclerosis and CVD. Adipose tissue is one of the main organs that mediates lipid metabolism during a process where excess energy can be stored in adipose tissue in the form of triglycerides. Lipid metabolism, especially lipoproteins, is altered in ESRD (Mesquita et al., 2010). Lipoproteins are exposed to a uraemic environment in ESRD and thus may be qualitatively and quantitatively altered. Little is known about the interaction between lipoproteins and adipose tissue in ESRD and their characterization may be important to clarify CVD in ESRD.

ESRD-associated dyslipidaemia is characterised by hypertriglyceridaemia, impaired HDL metabolism and accumulation of apo B-containing lipoproteins (Attman and Samuelsson, 2009). This ESRD-associated dyslipidaemia has been proposed to contribute to increased cardiovascular disease risk in ESRD. In addition, progression of renal disease may be accelerated by dyslipidaemia (Sahadevan and Kasiske, 2002).

Chapter 3 described the isolation of lipoprotein fractions from patients with ESRD as well as healthy controls. An atherogenic lipoprotein profile was observed in patients treated with different dialysis modalities. The lipoprotein subfractions VLDL, LDL and HDL were further investigated in the present study in order to determine their potential role on lipid metabolism in adipose tissue. To achieve this, gene expression of lipoprotein lipase (LPL), the rate-limiting

step in the catabolism of triglyceride-rich lipoprotein, hormone-sensitive lipase (HSL) the enzyme that hydrolyzes lipid, complement protein 3(C3) the main component for acylation stimulating protein and low density lipoprotein receptor (LDL-R) the main factor in LDL clearance were compared in VLDL-treated, LDL-treated and HDL-treated in human adipocyte culture. Little has been reported with regard to interaction of lipoproteins with adipose tissue in ESRD.

The present study was designed to test the hypothesis that uraemia alters lipoproteins such as VLDL, LDL and HDL. These abnormal uraemic lipoproteins have a different effect on adipose tissue lipid metabolism. The altered lipid metabolism in turn has the potential to contribute to the dyslipidaemia in ESRD.

4.2 Methods

4.2.1 Culture and differentiation of human preadipocytes (PromoCell)

Human white preadipocytes derived from subcutaneous adipose tissue of a female Caucasian subject (BMI 21; age 44 years) were obtained from PromoCell (PromoCell, UK). Preadipocytes were cultured in preadipocyte growth medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Lonza, Tewkesbury, UK) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Preadipocytes were seeded onto 12-well plates and grown until confluence. At confluence, cells were induced to differentiate (day 0) by incubation for 3 days in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (1:1) medium containing 32 μM biotin, 1 μM dexamethasone, 200 μM 3-isobutyl-1-methyl-xanthine, 100 nM insulin, 11 nM L-thyroxine (all from Sigma, Poole, Dorset, UK), 8 μM rosiglitazone (GlaxoSmithKline, Uxbridge, UK) and

100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After induction, cells were further maintained in feeding medium containing 3% fetal calf serum (FCS; Sigma), 100 nM insulin, 32 μM biotin and 1 μM dexamethasone until full differentiation. Differentiation into adipocytes was confirmed by observing the accumulation of lipid droplets under the microscope.

In a separate set of studies, adipocytes were incubated with lipoprotein fractions from different groups (Pre-haemodialysis, post-haemodialysis and peritoneal dialysis). All lipoprotein fractions in this set of studies were fractionated by self-generating iodixanol gradients following ultra centrifugation and filtration. The final concentrations of each fraction in the media were 50 mg protein/L as this concentration shown the absence of cytotoxicity (Masella et al., 2006). The media used for the cells as a control had their pre-incubation medium renewed. Cells were then collected after 4 and 24 h in 700 μl of TRI reagent.

4.2.2 Real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen, USA) and the RNA concentration determined from the absorbance at 260 nm. First strand cDNA was reverse transcribed from 0.5 μg of total RNA using an iScript first strand synthesis kit (BioRad, Hercules, USA) in a final volume of 10 μl. Real-time PCR amplification was performed in a final volume of 12.5 μl, containing cDNA (equivalent to 10 ng of RNA), optimising concentrations of primers, TaqMan probe FAM-TAMRA and a master mix made from qPCR core kit (Eurogentec, Seraing, Belgium) using a Stratagene Mx3005P instrument. PCR amplification was performed in duplicate using 96-well plates and the PCR cycling conditions

were as follows: 95 °C for 10 min followed by 40 cycles (95 °C for 15 s, 60 °C for 1 min). Blank controls without cDNA were run in parallel. POL2A was used as a reference gene. All samples were normalised to the the POL2A values and the results expressed as fold changes of Ct value relative to controls using the $2^{-\Delta\Delta ct}$ formula.

4.2.3 Statistical analysis

The statistical differences between the groups were evaluated through ANOVA followed by *post hoc* testing with Bonferroni correction. Differences were considered as statistically significant when $P < 0.05$.

4.3 Results

4.3.1 VLDL effect on gene expression

The PromoCell adipocytes were used at day 14 post-induction to examine the potential effect of VLDL on adipocytes. A 50mg protein/L concentration of lipoproteins was employed at two time points, 4 and 24 hrs.

As shown in figure 4.1 there was no effect found on LPL mRNA level at 4 h while at 24 h, the differences in LPL mRNA level among the groups were significant ($p < 0.001$). However, in the case of HSL, LDL-R and C3 there was no difference among the groups (Figure 4.2 to 4.4).

Moreover, VLDL treatment for 24 h led to a significant downregulation in LPL and LDL-R gene expression, with 2-fold and 2.5-fold response to Post-HD and PD, respectively ($p < 0.05$) (Figure 4.3). There was no effect found on C3 gene expression at both time points (Figure 4.4).

Comparison of VLDL treatments from the Pre-HD and Post-HD groups indicated that there was no difference in LPL, HSL, and LDL-R and C3 gene expression of the adipocytes (Figure 4.1 to 4.4).

4.3.2 LDL effect on gene expression

Gene expression of LPL, HSL, LDL-R and C3 in adipocyte cells was investigated by real-time PCR to examine the potential role of uraemic LDL on adipocytes.

At 4 h, the differences in LPL mRNA level among the groups were significant ($p < 0.05$). *Post hoc* analysis (Bonferroni correction test) showed that only the decrease observed for post haemodialysis group ($p < 0.05$).

At 24 h, the differences in LPL mRNA level among the groups were significant ($p < 0.001$). *Post hoc* analysis (Bonferroni correction test) showed that the decrease observed for post haemodialysis vs peritoneal group ($p < 0.001$) and also in Pre-HD vs Post-HD ($p < 0.05$) (Figure 4.5).

For HSL, the differences in the mRNA level among the groups were significant only at 4 h ($p < 0.05$). *Post hoc* analysis (Bonferroni correction test) showed that the decrease observed for Post-HD vs PD ($p < 0.05$) (Figure 4.6).

As shown in figure 4.7 there was no effect found on LDL-R mRNA level at 24 h while at 4 h, the differences in LDL-R mRNA level among the groups were significant ($p < 0.05$). LDL gene expression was downregulated significantly in the Post-HD group by 3.3-fold ($p < 0.05$) and and PD 2.5 fold ($p < 0.05$) (Figure 4.7) according to the *post hoc* analysis (Bonferroni correction test).

C3 mRNA level was differet among the groups were significant at 4 h ($p < 0.001$)

and 24 h ($p < 0.05$). *Post hoc* analysis (Bonferroni correction test) at 4 h showed that only the decrease observed for pre-HD ($p < 0.05$) and Post-HD ($p < 0.001$) vs healthy groups by 3.3 fold for each (Figure 4.8), while *post hoc* analysis (Bonferroni correction test) at 24 h showed that only the decrease observed in Pre-HD vs PD ($p < 0.001$).

4.3.3 HDL effect on gene expression

Gene expression of LPL, HSL, LDL-R and C3 in adipocyte cells was also investigated by real-time PCR to examine the potential role of uraemic HDL fraction on adipocytes.

At 4 h, the differences in LPL mRNA level among the groups were significant ($p < 0.001$). *Post hoc* analysis (Bonferroni correction test) showed that only the decrease observed for PD vs healthy group ($p < 0.001$) (Figure 4.9).

At 4 h, the differences in HSL mRNA level among the groups were significant ($p < 0.001$). *Post hoc* analysis (Bonferroni correction test) showed that only the decrease observed for PD vs healthy group achieved significance ($p < 0.001$) (Figure 4.10).

No difference seen on the LDL-R mRNA level when trated by HDL fractions (Figure 4.11).

For C3, the differences in the mRNA level among the groups were significant only at 4 h ($p < 0.05$). *Post hoc* analysis (Bonferroni correction test) showed that the decrease observed for PD vs healthy group ($p < 0.05$) (Figure 4.12).

There were no significant changes at 24 h for all studied genes.

Comparison of HDL treatments from the Pre-HD and Post-HD groups indicated that there was no difference in LPL, HSL, and LDL-R and C3 gene expression of the adipocytes (Fig. 4.9 to 4.12).

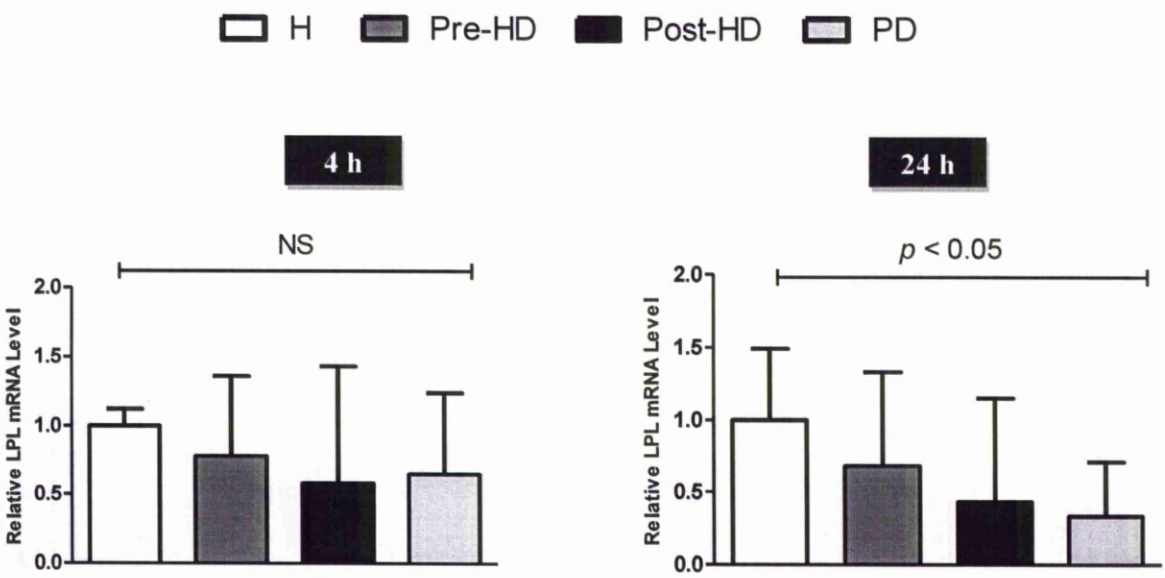

Figure 4.1: Effect of fractionated VLDL on LPL Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated VLDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. LPL mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with peritoneal dialysis VLDL resulted in a marked decrease on LPL expression at 24 h. Results are mean ± SD (n = 6/group).NS, not significant.

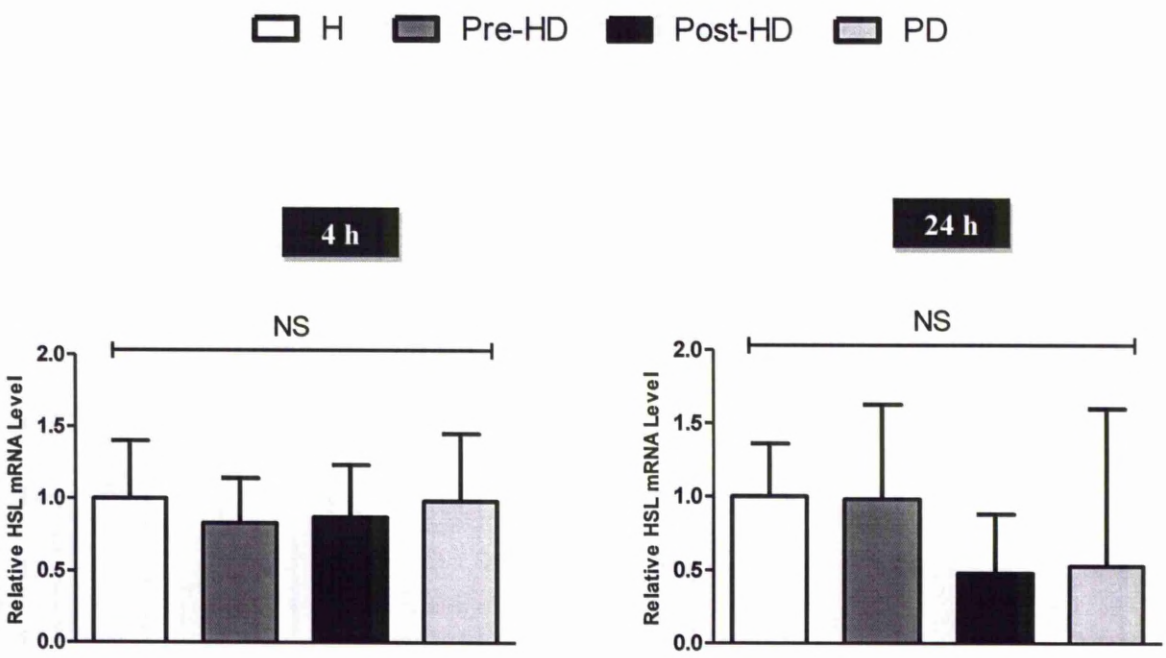

Figure 4.2: Effect of fractionated VLDL on HSL Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated VLDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. HSL mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Post-haemodialysis VLDL resulted in a marker decrease on HSL expression at 24 h. Results are mean ± SD (n = 6/group).NS, not significant.

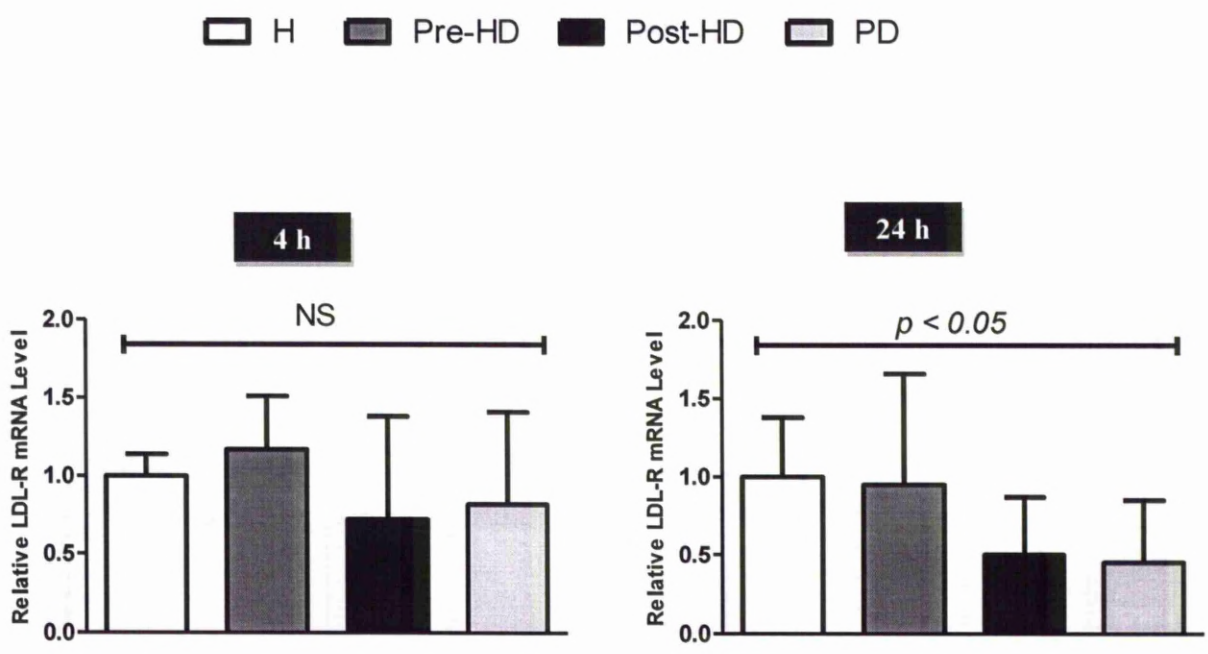

Figure 4.3: Effect of fractionated VLDL on LDL-R Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated VLDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. LDL-R mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Peritoneal and post-haemodialysis VLDL resulted in a marker decrease on LDL-R expression at 24 h. Results are mean ± SD (n = 6/group).NS, not significant.

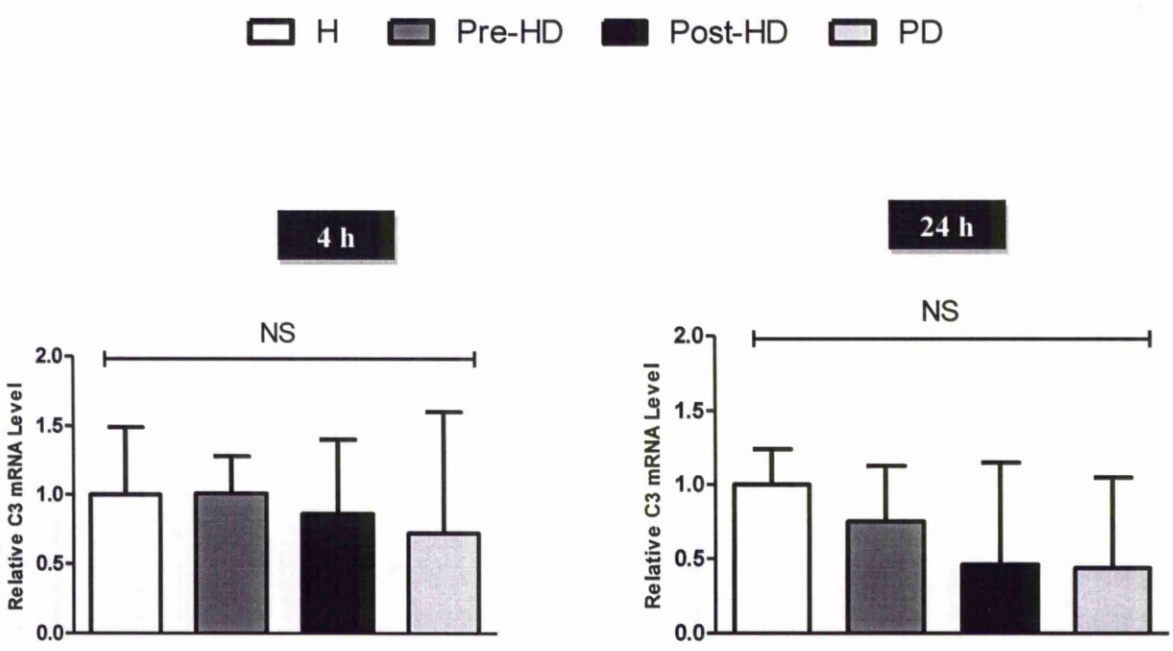

Figure 4.4: Effect of fractionated VLDL on C3 Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated VLDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. C3 mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with VLDL fraction from all groups did not alter gene expression for C3 in both 4 and 24 h. Results are mean ± SD (n = 6/group).NS, not significant.

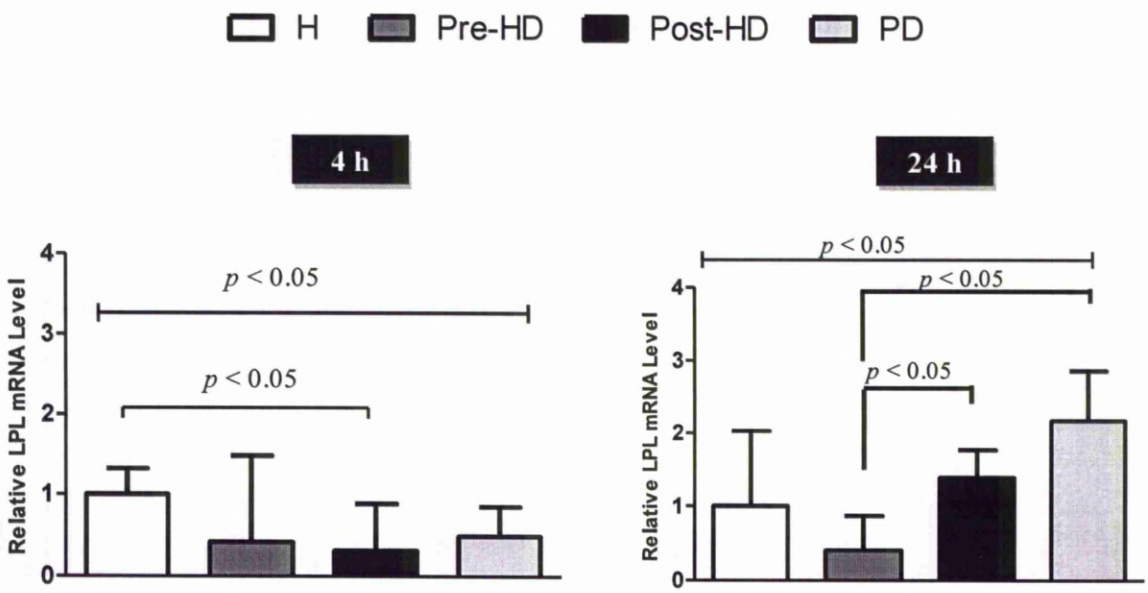

Figure 4.5: Effect of fractionated LDL on LPL Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated LDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. LPL mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Peritoneal and post-dialysis LDL resulted in a marker decrease on LPL expression in both 4 and 24 h. Results are mean ± SD (n = 6/group).

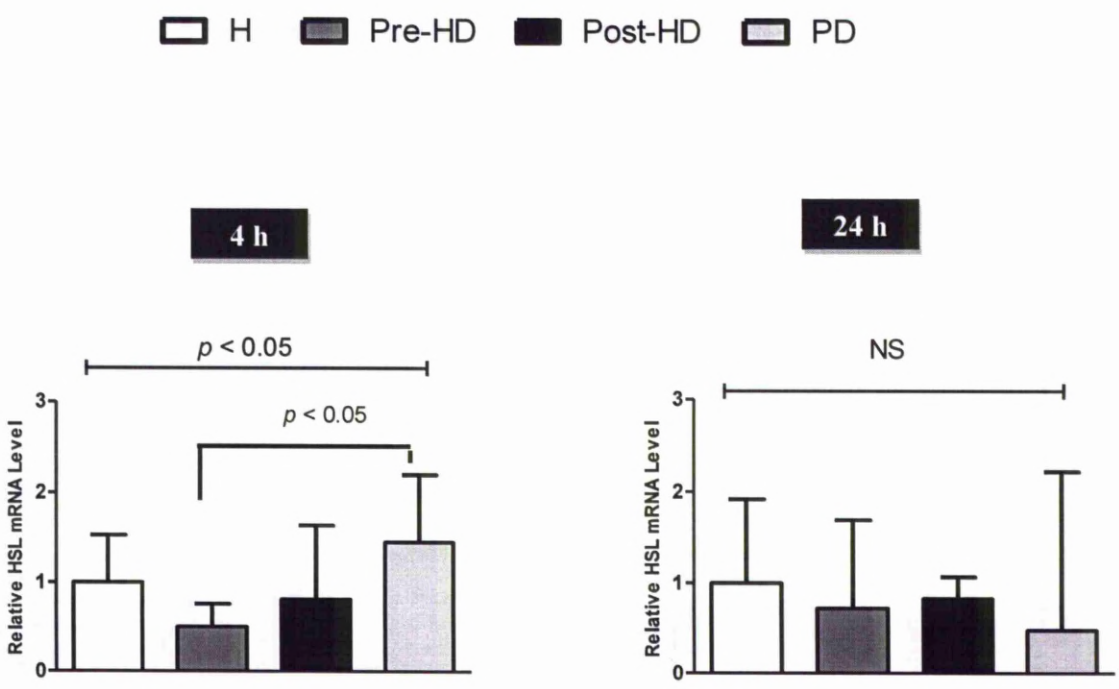

Figure 4.6: Effect of fractionated LDL on HSL Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated LDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. HSL mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Post-dialysis LDL resulted in a marker decrease on HSL expression at 4 h. Results are mean ± SD (n = 6/group).NS, not significant.

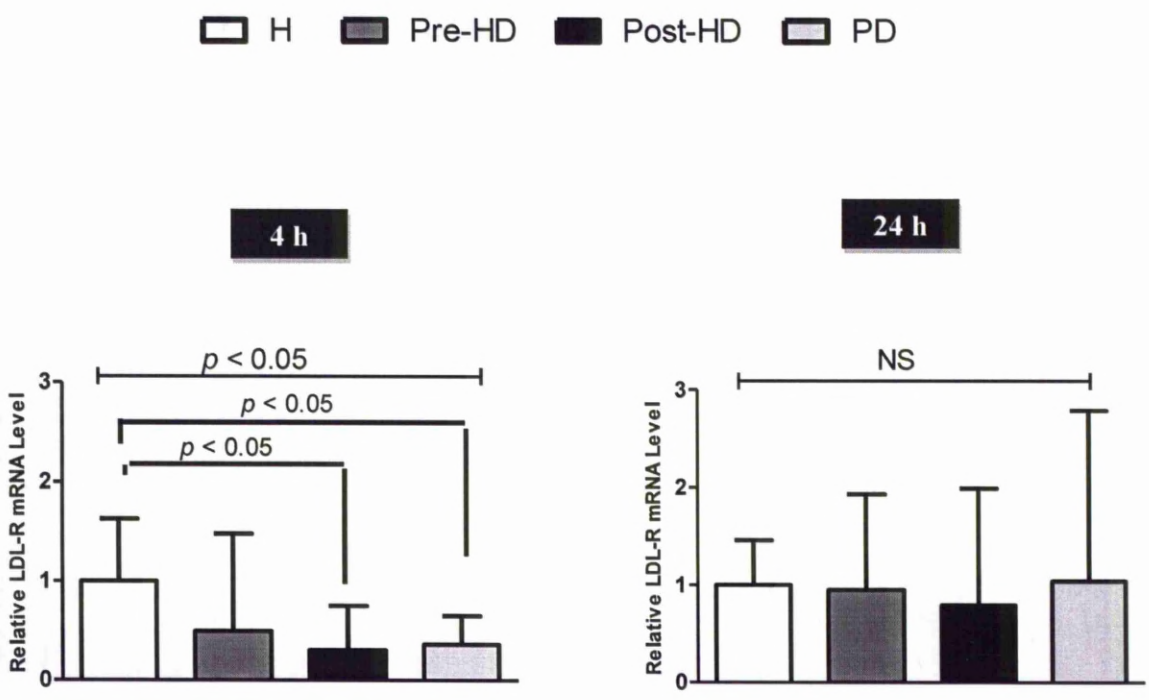

Figure 4.7: Effect of fractionated LDL on LDL-R Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated LDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. LDL-R mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Peritoneal and post-dialysis LDL resulted in a marked decrease in LDL-R expression after 4 h. Results are mean ± SD (n = 6/group).NS, not significant.

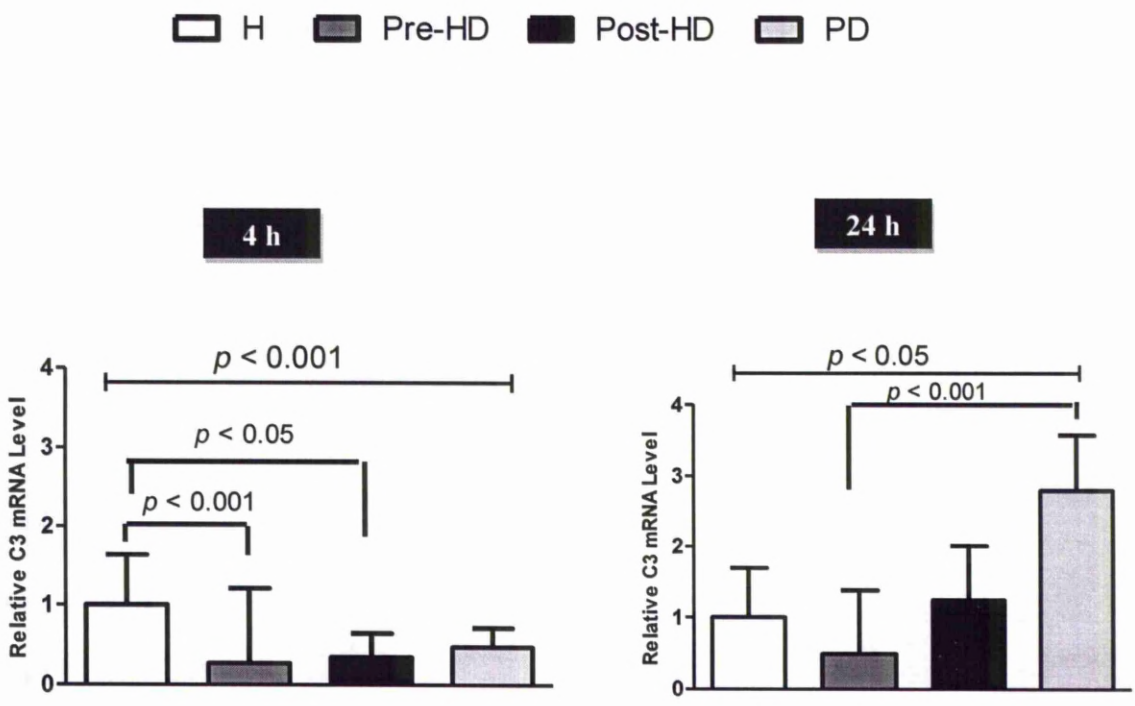

Figure 4.8: Effect of fractionated LDL on C3 Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated LDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. C3 mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with uraemic LDL resulted in a marker decrease on C3 expression at 4 h. Results are mean ± SD (n = 6/group).

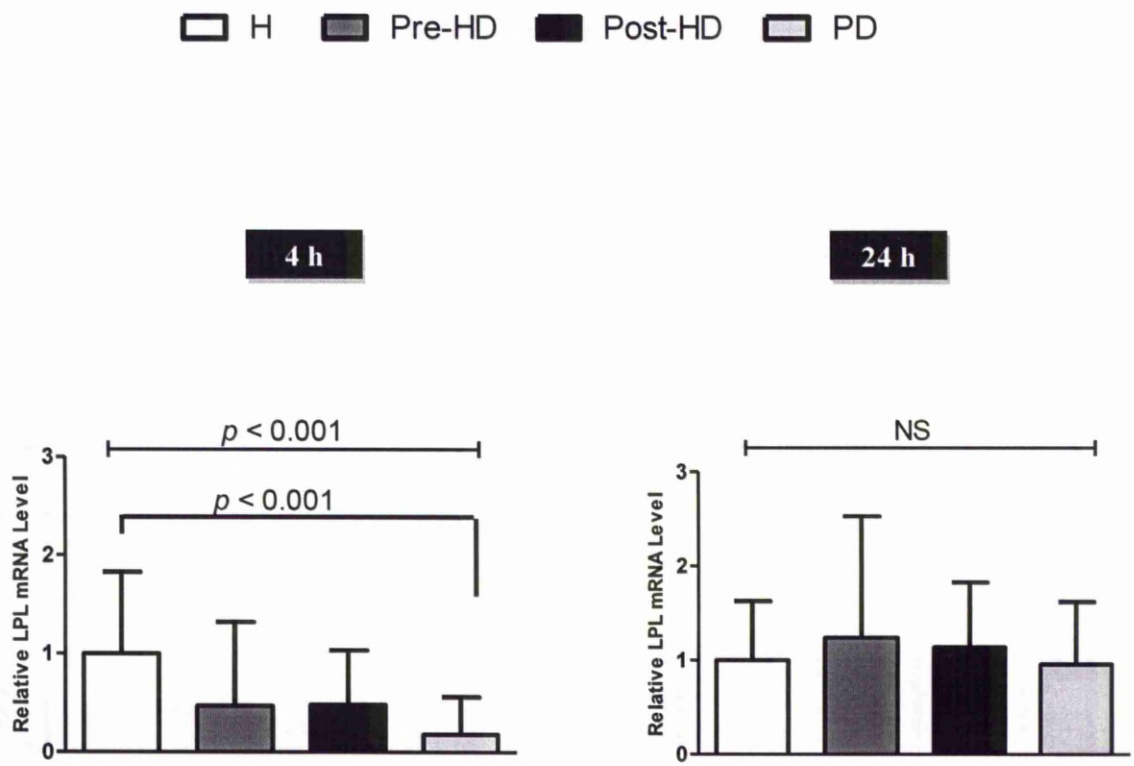

Figure 4.9: Effect of fractionated HDL on LPL Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated HDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. LPL mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Peritoneal dialysis HDL resulted in a marked decrease on LPL expression at 4 h. Results are mean ± SD (n = 6/group).NS, not significant.

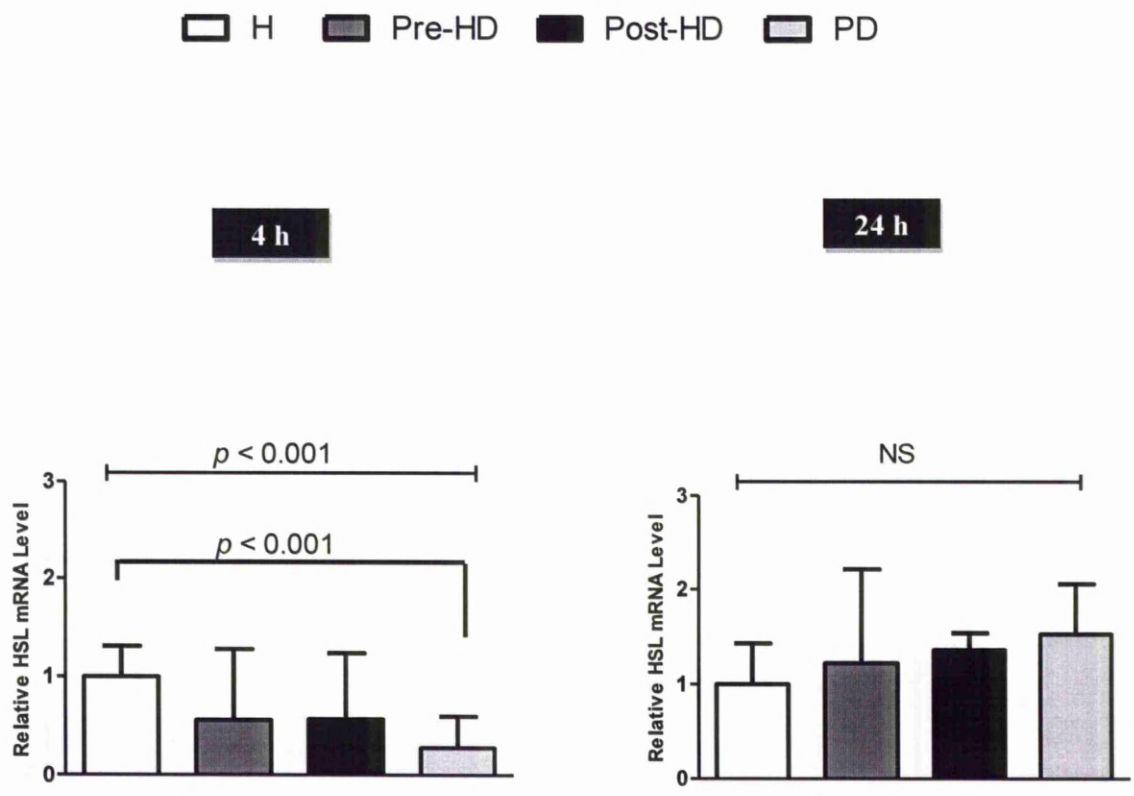

Figure 4.10: Effect of fractionated HDL on HSL Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated HDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. HSL mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Peritoneal dialysis HDL resulted in a marked decrease on HSL expression at 4 h. Results are mean ± SD (n = 6/group).NS, not significant.

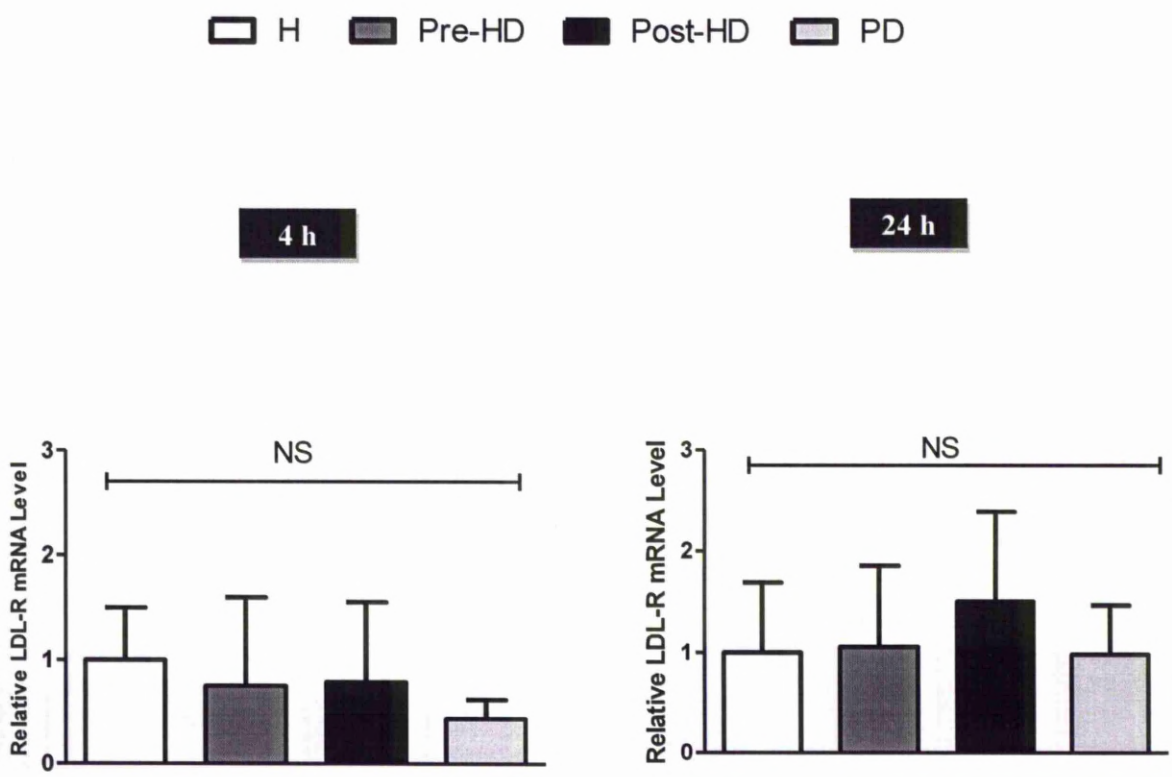

Figure 4.11: Effect of fractionated HDL on LDL-R Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated HDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. LDL-R mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with Peritoneal dialysis VLDL resulted in a marked decrease on LDL-R expression at 4 h. Results are mean ± SD (n = 6/group).NS, not significant.

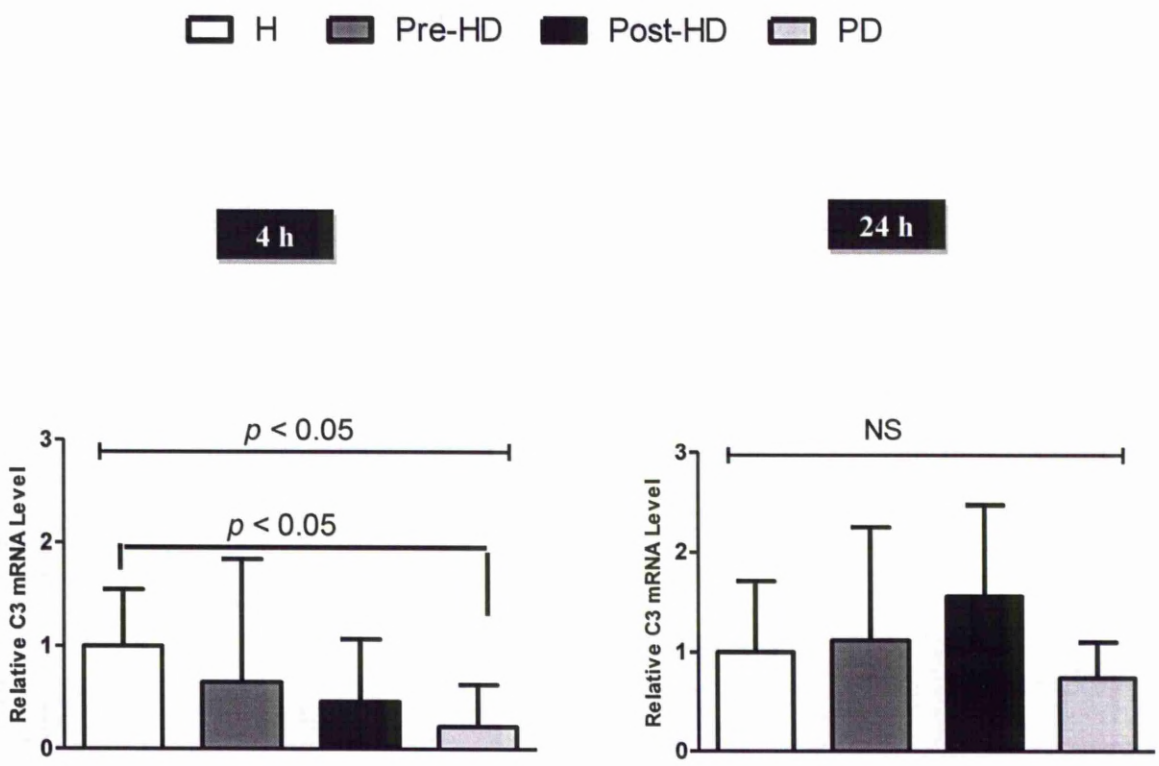

Figure 4.12: Effect of fractionated HDL on C3 Gene Expression in Human Adipocytes

Differentiated human adipocytes were exposed to 50 mg protein/L of fractionated HDL from healthy (H) and ESRD patients' pre and post dialysis for 4 and 24 hrs. C3 mRNA levels were normalised to human POLR2A and expressed relative to the healthy treated group. Treatment with peritoneal dialysis HDL resulted in a marked decrease on C3 expression at 4 h. Results are mean ± SEM (n = 6/group).NS, not significant.

4.4 Discussion

In this study normal adipocytes were exposed to lipoporteins from uraemic patients and healthy patients *in vitro*. This was done to assess whether there were any differences in the changes of gene expression of key proteins of lipid metabolism. Potentially these changes could play a part in the cardiovascular risk and dyslipidaemia of ESRD. The present study employed the lipoprotein fractions, VLDL, LDL and HDL, characterised in chapter 3. Adipose tissue is involved in lipid metabolism and linked to insulin resistance and type 2 diabetes. Moreover, adipose tissue is considered as an essential source for free fatty acids and therefore thought to be linked to many disease states (Arner, 2003).

LPL is a member of the lipase gene family and considered as the rate-limiting step in the hydrolysis of TG content found in the circulating chylomicron and VLDL which provides a supply of fatty acids to different tissues for either energy utilisation or storage as TG in the adipose tissue (Mead et al., 2002, Wang and Eckel, 2009). The results demonstrated that adipocyte LPL expression is downregulated to a greater extent using VLDL derived from dialysis patients when co-cultured. LPL is known to be regulated by post-translational control; therefore not seeing changes does not mean that there were no changes. mRNA changes, either increase or decrease, does not necessarily mean increase in protein or activity. It has been reported that LPL deficiency is associated with an increase in plasma TG, VLDL concentrations and impairment of chylomicron clearance in ESRD (Vaziri and Moradi, 2006). Low LPL activity has been observed in adipose tissue from ESRD (Goldberg et al., 1978). It has been previously reported that no

difference was noted in plasma LPL activity between haemodialysis and peritoneal dialysis (Shoji et al., 1991). VLDL fractions from pre-, post-HD and PD groups had more cholesterol compared with the healthy group (chapter 3) and VLDL cholesterol is considered as an independent risk factor for CVD (Shoji et al., 2001). ESRD animal studies have found a downregulation in LPL gene expression in heart, muscle and adipose tissue (Vaziri and Liang, 1996). Moreover, it has been proposed that reduction in LPL expression might lead to diminished physical activity, wasting syndrome and skeletal and cardiac myopathies due to the limited availability of free fatty acids (Vaziri and Liang, 1996). In the latter study, it has been reported that the effect of ESRD on LPL gene expression in adipose tissue is largely nonspecific and less of an effect when compared to skeletal muscle expression (Vaziri and Liang, 1996). It has been documented that inhibition of LPL leads to impairment of TG-rich lipoprotein, thereby leading to triglyceride accumulation in ESRD (Kaysen, 2006a). On the other hand, it has been suggested that low LPL activity is not a primary pathogenic factor for defective lipolysis in ESRD (Arnadottir, 1997).

Another studied gene, hormone-sensitive lipase, which hydrolyzes the stored TG in adipose tissue, is considered as the rate-limiting enzyme in adipose lipolysis (Kraemer and Shen, 2002). The results in this chapter show that HSL expression was lower when incubated with VLDL from pre-HD and PD groups when compared with the healthy group. The significance of inhibition of HSL may be either beneficial or harmful in terms of biology in ESRD. It would be protective if the main effect is to decrease overproduction of VLDL by the liver and therefore minimize hypertriglyceridaemia. On the other hand, it could be harmful if fatty

acids were not available for tissue energy purposes especially during the fasting state (Prinsen et al., 2003). However, other investigators have suggested that HSL activity was similar between healthy and some pathological states such as metabolic syndrome (Holm et al., 2000).

The hydrolysis of lipoprotein triglycerides takes place on the surface of the capillary endothelium. LPL is synthesized as an inactive, monomeric proenzyme form in the rough endoplasmic reticulum. The major sites for the synthesis of LPL are the adipose tissue, cardiac and skeletal muscles (Wang and Eckel, 2009). The proenzyme undergoes activation by sequential glycosylation and removal of a 24 amino acid peptide in the Golgi apparatus. Within the cell the active and inactive forms of the enzyme are stored in secretory vesicles which are translocated to the cell surface via a cAMP-mediated process (Mead et al., 2002). The precise mechanism for this transolcation of LPL across endothelial cells is currently unclear. However, recent study suggested that LPL binds to a newly discovered membrane-tethered protein on the luminal surface of endothelial cells, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein1 (GPIHBP1) (Beigneux et al., 2007). The enzyme is then released by an active process which can be up-regulated by heparin. After secretion, LPL becomes anchored to the capillary endothelial surface by binding to a specific heparan sulphate proteoglycan receptor. LPL synthesis and activity is regulated by various mechanisms like nutritional status and hormonal action. These mechanism involve post-transcriptional regulation including mRNA stability, translation, protein degradation, processing, secretion,translocation to the site of action, and competitive inhibition by products (Mead et al., 2002). Insulin increases LPL

mRNA levels in adipocytes without any changes in the rate of gene transcription. Also, fasting result in reduction in LPL activity and has been proposed to prevent of transfer from the ER to the Golgi (Wang and Eckel, 2009). High concentration of NEFA has been shown to lead to LPL displacement (Olivecrona et al., 1997).

HSL activity is induced by cAMP-dependent PKA pathway and protein kinase G (PKG). in the basal state, HSL is distributed throughout the cytosol upon its activated and rapidly translocated to the surface of lipid droplets (Lass et al., 2011).

LDL-R mediates cholesterol uptake from the circulation. In this study, LDL-R was downregulated with post-HD and PD VLDL. Other groups have found that LDL-R expression did not differ between ESRD experimental animals and healthy controls (Liang and Vaziri, 1997a).

The results in the present studies indicated that treatment of adipocytes with uraemic lipoproteins can downregulate gene expression of key lipolysis enzymes. One could speculate that in situations where lipoprotein was not efficiently cleared from plasma, as in ESRD, by lipoprotein lipase hydrolysis, the resulting TG-rich remnant lipoproteins could be catabolised in the liver and stimulate the assembly and secretion of nascent VLDL. This mechanism might be of particular importance during the postprandial period, with VLDL concentrations being stimulated and increased in the fasting state. Remnant particles are also more atherogenic as they can be cleared by scavenger receptors on macrophages, a direct link to atherosclerosis. Remnants are also cleared more rapidly than other lipoprotein fractions (Itabe et al., 2011).

There has been accumulated evidence that TG-rich lipoprotein hydrolysis is altered in ESRD (Arnadottir, 1997). Uraemic lipoprotein fractions were more likely to be either structurally or functionally modified or both. VLDL and IDL have been shown to affect the *in vitro* lipolysis rates in ESRD (Lee et al., 2002). In ESRD, altered lipoprotein metabolism can be seen more clearly in the apolipoprotein profile and the lipoprotein composition than in lipoprotein concentrations (Kastarinen et al., 2009a). ESRD-dyslipidaemia has been shown to be associated with CVD and renal disease. There are many factors that contribute to dyslipidaemia in ESRD including decreased activity of lipolytic enzymes and alterations in the lipoprotein substrate and diminished uptake of lipoproteins (Chan et al., 2008, Vaziri and Moradi, 2006).

Numerous studies have shown that atherogenic lipoproteins stimulate expression of inflammatory mediators *in vitro*. MCP-1 gene expression was upregulated in endothelial cell culture by diabetic IDL (Maeno et al., 2000). It has been reported that during acute-phase response, HDL becomes a pro-inflammatory molecule (Van Lenten et al., 1995). Moreover, pro-inflammatory HDL was shown to stimulate TNF-α and chemotaxis in monocytes (Skaggs et al., 2010). It has been found that lipoproteins remain for longer periods in the circulation in ESRD and are likely to undergo modification in their structure (Quaschning et al., 2001). One of the consequences of modification is that receptor recognition is altered (Kramer-Guth et al., 1997). Modified lipoproteins have also been shown to be a poor substrate for lipolytic enzymes (Arnadottir et al., 1996). VLDL overproduction in combination with reduced clearance may result in the formation of small dense LDL, the atherogenic form. It has been suggested that the anti-

oxidant protective capacity of HDL is decreased in ESRD and that HDL may not prevent LDL oxidation (Morena et al., 2000).

The results in this thesis have showed that the VLDL with LDL fractions had an inhibitory effect on adipocyte gene expression. There is accumulated evidence that VLDL is associated with atherosclerosis acceleration by upregulating pro-inflammatory mediators. VLDL and oxidised LDL have been reported to stimulate cytokine expression including TNF-α (Ares and Stollenwerk, 2006). Moreover, VLDL has been shown to stimulate the production of the pro-inflammatory cytokine, interlukin-1β in human peripheral blood monocyte-derived macrophage (Stollenwerk et al., 2005) and increased expression of adhesion molecules in endothelial cells (Ares and Stollenwerk, 2006). ESRD patients in this study have been shown to have higher levels of VLDL-cholesterol, particularly those on peritoneal dialysis (see chapter 3).

In conclusion, expression of lipolysis enzymes and regulatory proteins of lipid metabolism are affected when treated with uraemic lipoproteins *in vitro*. These data may suggest that uraemic lipoproteins may play a role in dyslipidaemia in ESRD. It will be important to demonstrate similar findings in experimental ESRD and also at the protein expression level.

CHAPTER 5

ADIPOKINE GENE EXPRESSION OF HUMAN ADIPOCYTES IN ESRD

5.1 Introduction

Traditionally adipose tissue was considered to be mainly a storage depot for dietary triglycerides. However, it is now recognised to be an endocrine organ and has become an active area of research. The adipocytes (and other cells in adipose tissue) produce cytokines, chemokines, growth and complement factors which may have a role in ESRD (Chudek and Wiecek, 2006, Trayhurn, 2007). The endocrine function of adipose tissue involves the production and secretion of a large number of hormones, several of which are the focus of the present study. The best known among these factors is leptin, an adipocyte-derived hormone that acts in the hypothalamus and peripheral tissues to decrease food intake, increase energy expenditure, and other cellular functions. In addition to leptin, many other factors have been identified including tumour necrosis factor (TNF-α), adiponectin, plasminogen activator–inhibitor, interleukin 6, resistin, transforming growth factor-β, adipsin and retinol-binding protein (Ahima and Osei, 2008, Trayhurn and Wood, 2004).

Leptin is an important adipocyte product. Leptin has many functions elicited through specific interaction with its receptor (LR) found in most tissues. Mainly, leptin inhibits food intake (Ahima, 2006, Trayhurn and Beattie, 2001). Leptin levels are high in chronic renal failure (CRF) patients especially those undergoing PD (Heimburger and Stenvinkel, 2005) and its role in the nutritional status has been investigated (Kwan and Beddhu, 2007). Leptin gene expression is decreased in chronic renal failure (Nordfors et al., 1998) even though circulating concentrations are increased suggesting a major role for the kidneys in the degradation and elimination of leptin.

Adiponectin is produced solely by mature adipocytes and is a member of soluble defence collagen family (Ahima, 2006, Costacou and Orchard, 2008). Adiponectin has been observed in three different forms; low, middle and high molecular weight (Sowers, 2008). The high molecular weight is considered the active form (Wiecek et al., 2002). Adiponectin has anti-atherogenic, anti-inflammatory and insulin sensitizing functions (Costacou and Orchard, 2008). Adiponectin inhibits oxidised LDL-mediated cell proliferation, lipid accumulation in monocyte-derived macrophages and transformation of macrophages into foam cells (Chudek and Wiecek, 2006). In contrast to leptin, adiponectin is negatively associated with WAT mass (Ahima, 2006). Surprisingly although low plasma adiponectin level is associated with CAD and type 2 diabetes, it is increased in uraemia suggesting that the kidney may again be an important site for degradation and elimination (Chudek and Wiecek, 2006, Huang et al., 2004).

Interleukin-6 (IL-6), a pro-inflammatory marker, is produced by adipocytes as well as other cells (Ahima and Osei, 2008). It is a protein 22 to 27 kDa and induced by many factors including oxidative stress (Stenvinkel et al., 2005). Together with IL-6 and Monocyte Chemoattractant Protein -1 (MCP-1), IL-6 is considered important for both acute and chronic inflammation (Wozniak et al., 2009). IL-6 has been found to have a significant relationship with plasma high sensitivity C-reactive protein (hsCRP) in dialysis patients (Kwan and Beddhu, 2007). IL-6 and MCP-1 plasma levels are increased in ESRD (Stenvinkel et al., 2005, Taskapan et al., 2007, Wang et al., 2008b).

Circulating adipokine concentrations are altered in ESRD with differences between renal replacement therapies (Wiecek et al., 2002). We hypothesised that

the uraemic environment with retention of 'uraemic toxins' in ESRD results in adipose tissue dysfunction affecting not only lipoprotein metabolism but also adipokine production. The potential abnormalities in adipokines in ESRD could be a further possible link to CVD. This chapter is aimed specifically at characterising adipokines secretion in ESRD to determine if these changes could help explain the association between ESRD and CVD. The gene expression of key adipokines, adiponectin and leptin, and the proinflammatory adipokines IL-6 and MCP-1 were investigated using an *in vitro* human adipocyte culture system in the presence of serum obtained from ESRD patients expected to contain uraemic toxins.

5.2 Methods

5.2.1 Culture and differentiation of human primary preadipocytes

5.2.1.1 PromoCells

Human white preadipocytes derived from subcutaneous adipose tissue of a female Caucasian subject (BMI 21; age 44 years) were obtained from PromoCell (PromoCell,UK). Preadipocytes were cultured in preadipocyte growth medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Lonza, Tewkesbury, UK) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Preadipocytes were seeded onto 12-well plates and grown until confluence. At confluence, cells were induced to differentiate (day 0) by incubation for 3 days in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (1:1) medium containing 32 μM biotin, 1 μM dexamethasone, 200 μM 3-isobutyl-1-methyl-xanthine, 100 nM insulin, 11 nM L-thyroxine (all from Sigma,

Poole, Dorset, UK), 8 μM rosiglitazone (GlaxoSmithKline, Uxbridge, UK) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After induction, cells were further maintained in feeding medium containing 3% fetal calf serum (FCS, Sigma UK), 100 nM insulin, 32 μM biotin and 1 μM dexamethasone until full differentiation. Differentiation into adipocytes was confirmed by observing the accumulation of lipid droplets under the microscope.

5.2.1.2 SGBS Cells

Simpson-Golabi-Behmel syndrome (SGBS) human preadipocytes were maintained and differentiated into adipocytes. Cells were cultured in 12-well plates (20,000 cells/well) and maintained in DMEM/Ham's F12 (1:1 v/v) medium (Invitrogen, Paisley, UK) containing 10% (v/v) foetal calf serum (FCS, Sigma, UK), 33 μM biotin, 17 μM pantothenate, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2/95% air. Once confluent, differentiation was induced by incubating the cells in FCS-free induction media containing 0.25 μM dexamethasone, 0.5 mM isobutyl IBMX, 10 nM insulin, 200 pM triiodothyronine (T3), 0.1 μM cortisol (Sigma) and 2 μM rosiglitazone (GlaxoSmithKline, UK) for 4 days. The cells were then fed every 3 days in feeding medium (induction medium without dexamethasone, IBMX and rosiglitazone). Differentiated SGBS cells, 10-12 days post-induction, were treated with serum, and control cells were incubated in feeding medium. The cells were harvested in 500 μl of Trizol (Invitrogen) and the medium collected; both were frozen at −80°C until required for analysis.

5.2.2 Treatment with uraemic sera

To examine the effect of uraemic sera on adipokines expression and secretion, fully differentiated adipocytes were incubated with serum or plasma, at day 12-14 post-induction for 4 and 24 h. Wells incubated with adipocyte medium, only for the same period were used as controls. Incubations at each time-point were performed in 6-8 replicate. Cells and the culture medium were collected, centrifuged at 1000 rpm for 10 min to remove cell debris, and the supernatant was stored at −20 °C until further analysis.

5.2.3 Real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen, USA) and the RNA concentration determined from the absorbance at 260 nm. First strand cDNA was reverse transcribed from 0.5 μg of total RNA using an iScript first strand synthesis kit (BioRad, Hercules, USA) in a final volume of 10 μl. Real-time PCR amplification was performed in a final volume of 12.5 μl, containing cDNA (equivalent to 10 ng of RNA), optimised concentrations of primers, TaqMan probe FAM-TAMRA and a master mix made from qPCR core kit (Eurogentec, Seraing, Belgium) using a Stratagene Mx3005P thermocycler. PCR amplification was performed in duplicate using 96-well plates and the PCR cycling conditions were as follows: 95 °C for 10 min followed by 40 cycles (95 °C for 15 s, 60 °C for 1 min). Blank controls without cDNA were run in parallel. POL2A was used as a reference gene. All samples were normalised to the POL2A values and the results expressed as fold changes of Ct value relative to controls using the $2^{-\Delta\Delta ct}$ formula.

5.2.4 Measurement of adipokines

Adiponectin were measured in serum and cell culture medium using a human adiponectin ELISA kit (R&D System, Inc., UK) according to the manufacturer's instructions. The sensitivity was >50 ng/ml.

High molecular weight adiponectin was measured in serum and using a human adiponectin ELISA kit (Alpco, USA) according to the manufacturer's instructions. The sensitivity was > 0.19 ng/ml.

IL-6 was measured in serum using a human IL-6 ELISA kit (PEPROTEC, UK) according to the manufacturer's instructions. The sensitivity was > 50 pg/ml.

Leptin was measured in serum and using a human leptin ELISA kit (RayBiotech inc., USA) according to the manufacturer's instructions. The sensitivity was > 6 pg/ml.

5.2.5 Cell viability assessment

Cell viability was determined from the release of lactate dehydrogenase (LDH) into cell culture medium after treatment with serum using a spectrophotometric assay kit (Roche Diagnostics GmbH, Mannheim, Germany). LDH levels were measured by a spectrophotometer at 492 nm and 620 nm at room temperature.

5.2.6 Statistical analysis

Data are expressed as means ± SEM. Student's unpaired *t*-test was used for two groups' comparison. The statistical differences between more than two groups were evaluated through ANOVA followed by *post hoc* testing with Bonferroni correction. Differences were considered as statistically significant when $P < 0.05$.

5.3 Results

5.3.1 Cell viability assessment

In the initial set of investigations, various concentrations of serum and plasma were tested with regard to cytotoxicity determined by LDH release. The following groups were examined: healthy, pre-haemodialysis, post-haemodialysis using serum and plasma at the following volume: 10, 50, 100, 150 and 300 µl. Incubation of adipocytes with plasma resulted in clotting in the well (Table 5.1). To overcome the clotting problem, EDTA was added to the culture. The effects on cell viability were measured after 24 h incubation with different doses of potassium-EDTA dissolved in the normal growth medium (DMEM). The results of the measurements of cell viability (LDH tests) are shown in Table I. The data show, that potassium-EDTA concentration up to 2.5 mg/ml showed significant toxicity ($p < 0.0001$). Serum was found to have the lower cytotoxicity effect compared to plasma and that cytotoxicity increased with increasing volume of serum (Figure 5.2).

5.3.2 Dosage response to human serum levels

Varying proportions of human serum (expressed as percentage values) were added to SGBS adipocytes in order to assess the levels required to elicit a response in adipokine gene expression whilst minimising the levels of cell cytotoxicity. Gene expression levels of adiponectin and IL-6 were chosen as markers to optimize the *in vitro* culture assay system. As can be seen in figure 5.3, serum concentrations 0.5% and 5% resulted in measurable changes in gene expression levels and produced minimal levels of cytotoxicity and were used subsequently throughout

as low and high dose levels.

well content	1 h	2h	3h	6h	24 h
20 µl serum + serum free media					
30 µl serum + serum free media					
40 µl serum + serum free media					
50 µl serum + serum free media					
60 µl serum + serum free media					
70 µl serum + serum free media					
80 µl serum + serum free media					
90 µl serum + serum free media					
100 µl serum + serum free media					
75 µl plasma + serum free media	clot	clot	clot	clot	clot
100 µl plasma + serum free media		clot	clot	clot	clot
150 µl plasma + serum free media					
300 µl plasma + serum free media					
FCS free DMEM					

Table 5.1 Comparison between effects of serum vs plasma on the cultured adipocytes

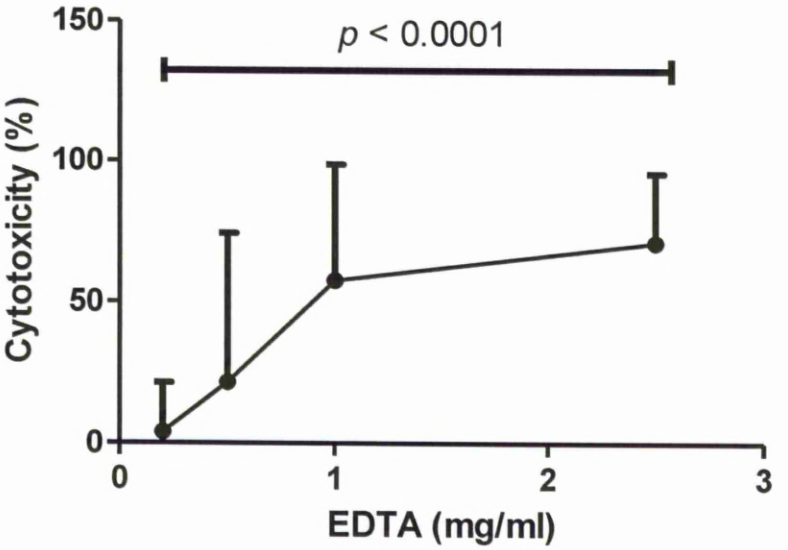

Figure 5.1 Toxic effects in normal adipocyte culture incubated for 24 h with different doses of k-EDTA

Differentiated human adipocytes were exposed to potassium-EDTA for 24 hrs. Results are mean ± SEM (n = 3). NS,

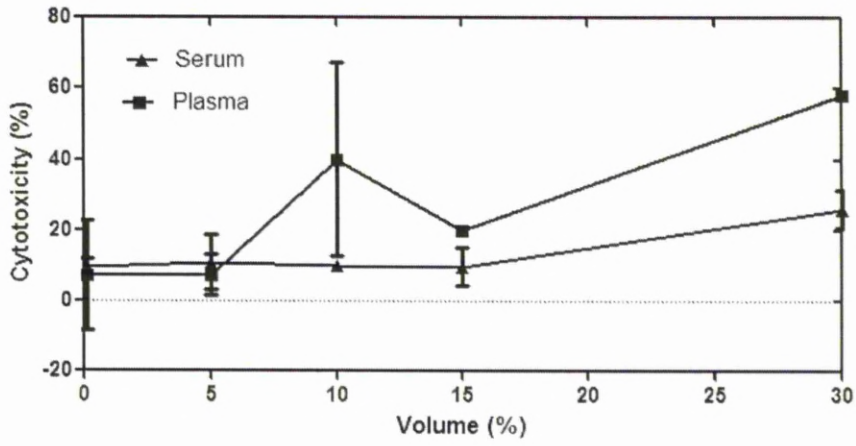

Figure 5.2: Cytotoxicity effect of various volumes of normal and uraemic serum and plasma on adipocyte cell cultures

At the end of the period exposure of uraemic serum and/or plasma, the cytotoxicity was evaluated with lactate dehydrogenase (LDH) release into the medium. Cytotoxicity values are represented as a percentage of the high control. Results are percentage ± SEM and all evaluated samples were performed in triplicate. Plasma was obtained from blood samples collected into EDTA.

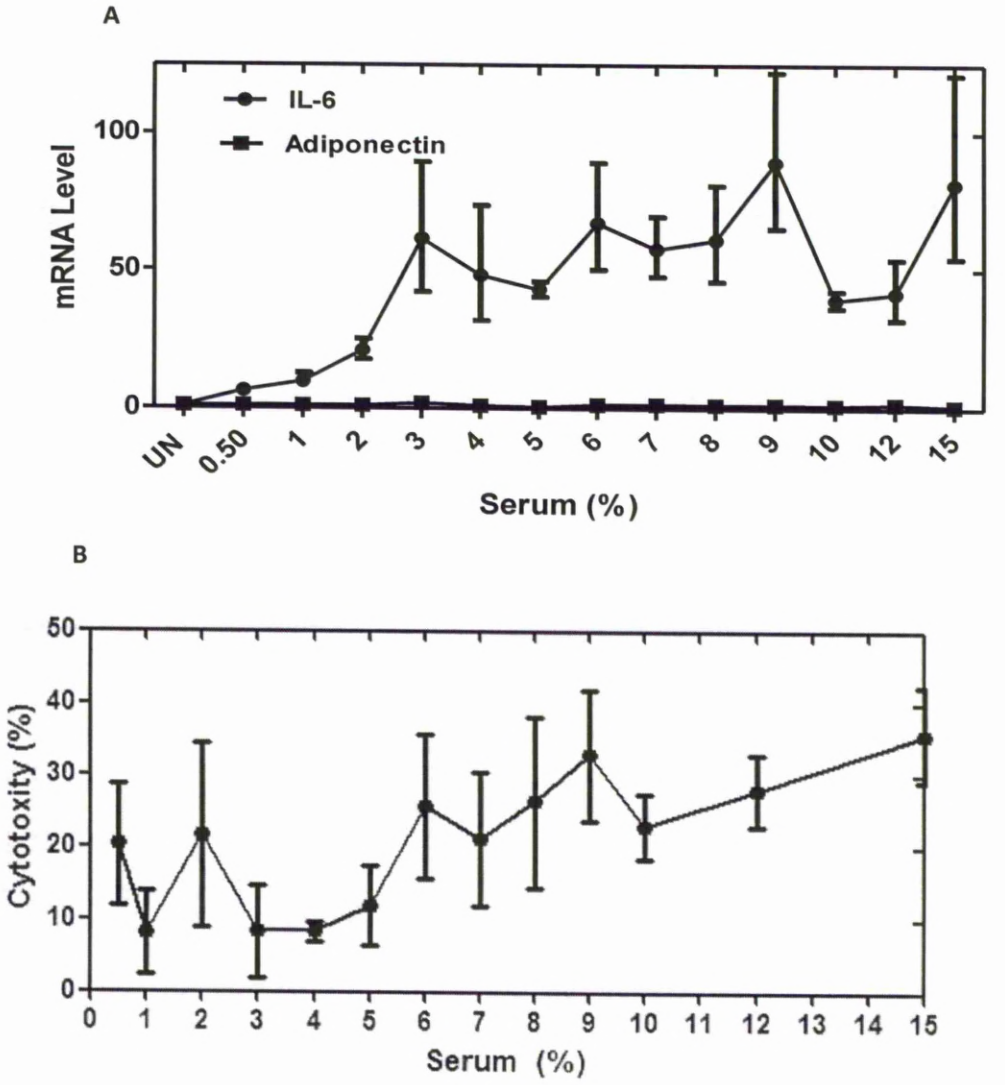

Figure 5.3**: Gene expression of IL-6 and Adiponectin in human adipocytes in response to serum treatment**

Differentiated SGBS adipocytes were incubated with human serum for 24 h. Cells and media were collected at the indicated time points. Measurement of IL-6 and adiponectin mRNA (A), was assessed by real-time PCR (normalised to POL2A compared to the untreated control group); (B) cytotoxicity effect. Data is presented as means ± SEM.

5.3.3 Serum total adiponectin and high molecular weight concentrations

The differences in serum high molecular weight adiponectin levels among the groups were significant ($p < 0.0001$). Serum high molecular weight adiponectin concentrations in ESRD patients were higher than those in healthy subjects. PD group presented higher values than HD groups (Pre and Post) (8.02 ± 5.14 μg/mL vs 3.03 ± 1.95 μg/mL; 3.57 ± 2.44 μg/mL respectively). *Post hoc* analysis (Bonferroni correction test) showed that the increase observed for PD vs H ($p < 0.0001$), PD vs Pre-HD ($p < 0.05$) and PD vs Post-HD ($p < 0.01$) (Figure 5.4A).

For total adiponectin, the difference among the groups was significant ($p < 0.001$). Both ESRD patients (HD and PD) were higher than those in healthy subjects. Also, PD group presented higher values than HD groups (Pre and Post) (13.7 ± 3.04 μg/mL vs 7.95 ± 1.44 μg/mL; 6.73 ± 1.2 μg/mL respectively) (Figure 5.4B). *Post hoc* analysis (Bonferroni correction test) showed that the increase observed for PD vs H ($p < 0.001$) and PD vs Post-HD ($p < 0.05$) achieved significance (Figure 5.4B).

5.3.4 Measurement of adiponectin gene expression in human adipocytes

The PromoCell adipocytes were used at day 14 post-induction to examine the potential effect of uraemic serum on adipocytes. 0.5 % and 5 % of serum were employed at two time points, 4 and 24 hrs.

The differences in adiponectin mRNA levels among the groups at 4 h with low dose were significant ($p < 0.0001$) as adiponectin gene expression was down-regulated 2-fold, 2.5-fold and 3-fold in the pre-HD, post-HD and PD groups, respectively (Figure 5.5). However, at 24 hr, adiponectin gene expression was

changed. *Post hoc* analysis (Bonferroni correction test) showed that the decrease observed for Post-HD vs H ($p < 0.0001$) and PD vs H ($p < 0.0001$) (Figure 5.5).

Comparison of serum treatments between the pre-HD and post-HD indicated that there was no significant change in adiponectin gene expression.

5.3.5 Measurement of adiponectin release by human adipocytes

As shown in figure 5.6, total adiponectin was measured. However, only changes noted with low dose at 4 h with no significant change was noted at 24 h.

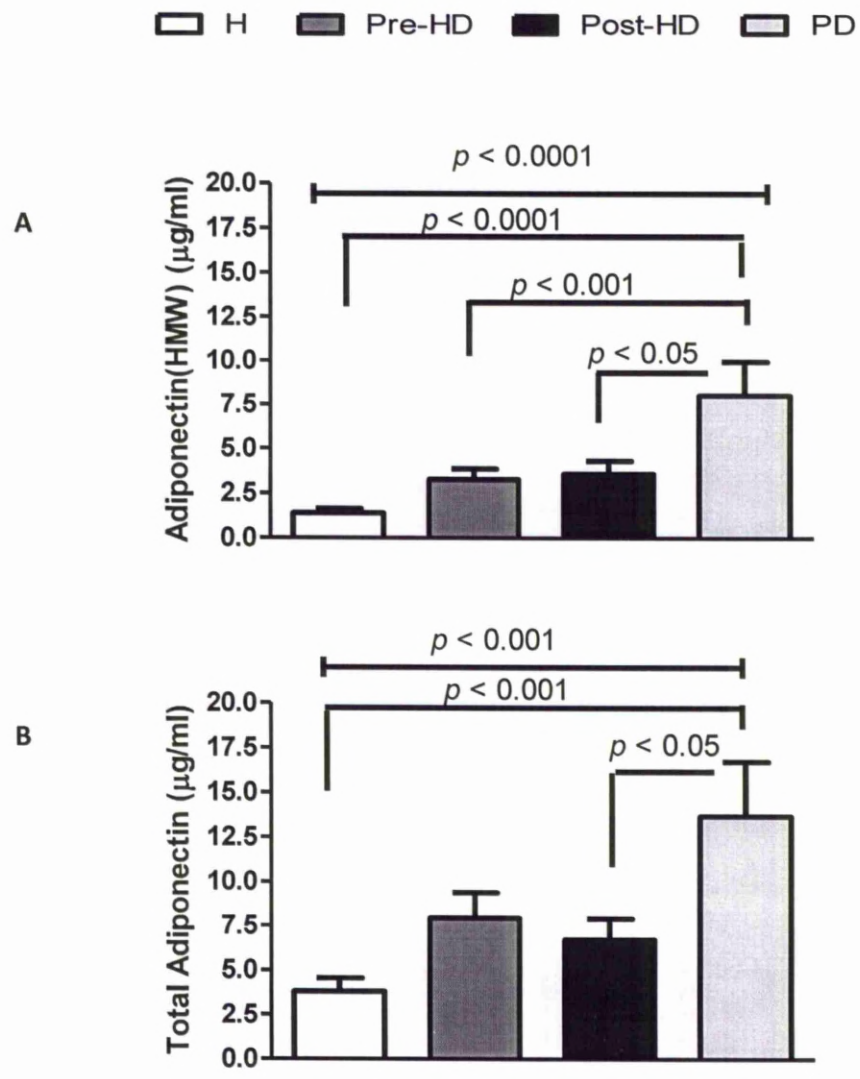

Figure 5.4 Circulating adiponectin levels in human subjects.

Serum concentrations of high molecular weight adiponectin (A) and total adiponectin (B) in healthy control subjects (white bars), ESRD patients pre-haemodialysis, Pre-HD (grey bars), ESRD patients post-haemodialysis, Post-HD (black bars) and ESRD patients peritoneal dialysis, PD (light grey bars). Values are means ± SEM ($n = 9$ for control group, $n = 11$ for HD group and $n = 7$ for PD group).

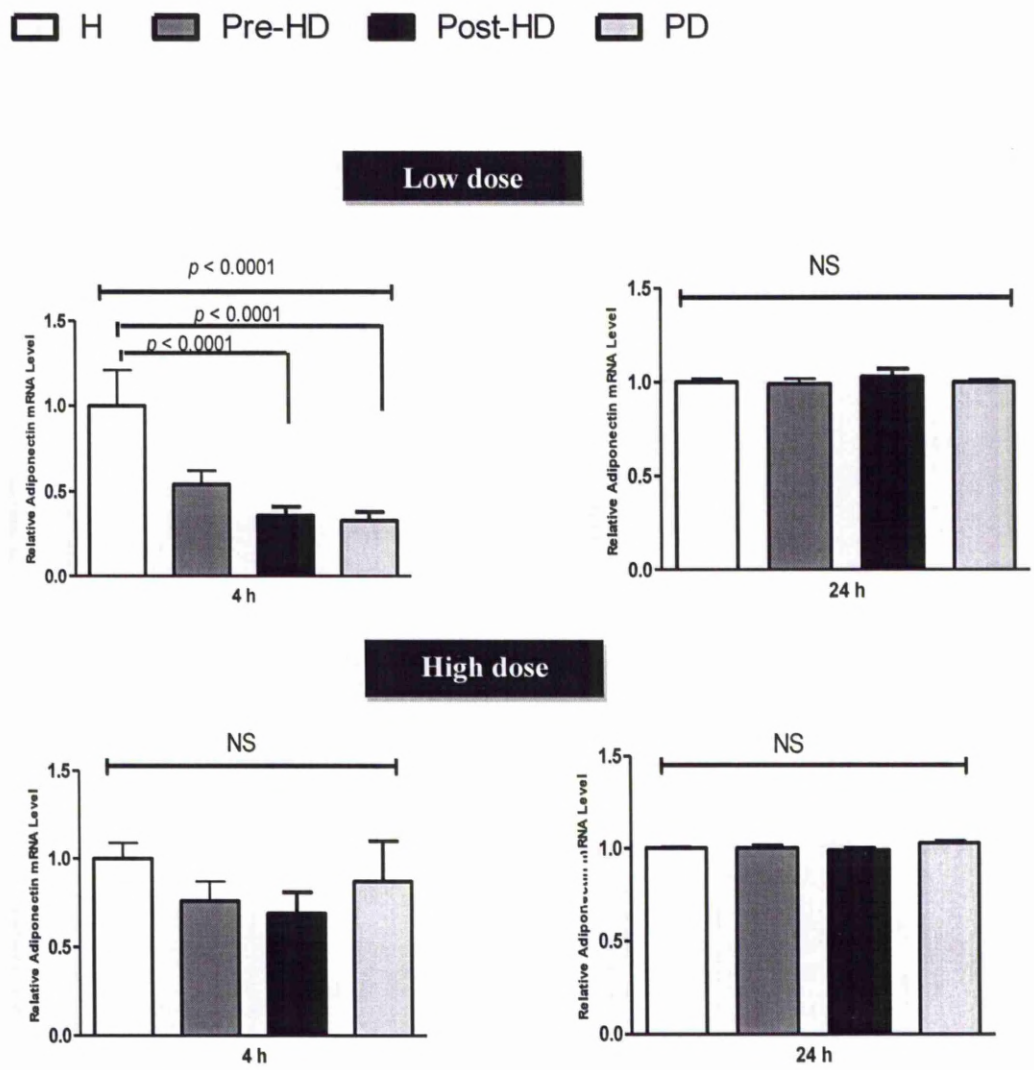

Figure 5.5 Adiponectin gene expression levels in adipocytes treated with uraemic serum

Differentiated human adipocytes were exposed to Low dose (upper panel) and to high dose (lower panel) of human pooled serum from healthy (H) and ESRD groups pre and post dialysis for 4 and 24 hrs. Adiponectin mRNA levels were normalised to human POLR2A and expressed relative to untreated cells. Results are mean ± SEM (n = 6/group). NS, not significant.

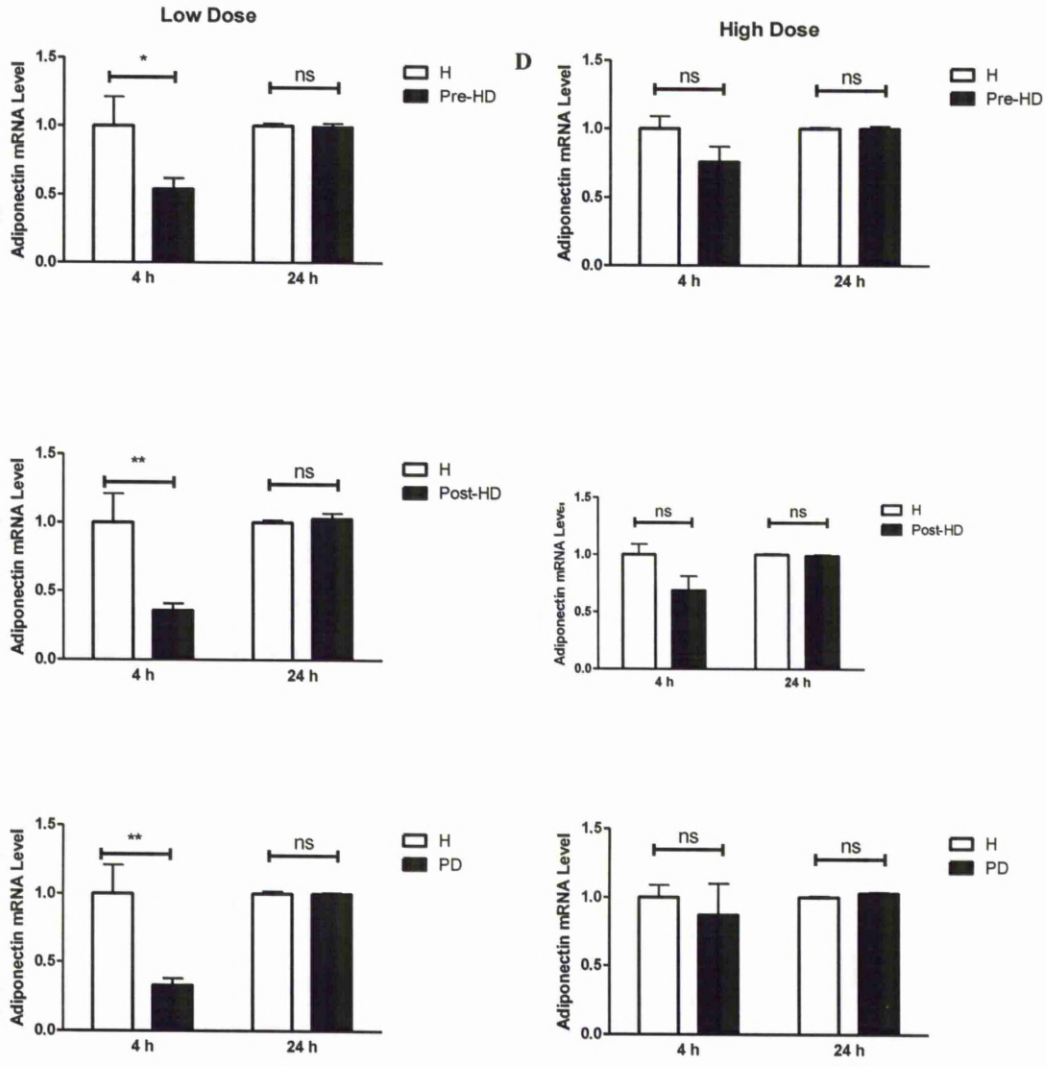

Figure 5.6 Release of adiponectin by human adipocytes treated with uraemic serum

Adiponectin secretion by human adipocytes in culture. **A, B** and **C** with Low dose. **D, E** and **F** with high dose. The adiponectin were measured by ELISA in culture media of cells after the incubation of serum with low dose a and high dose b for 4 and 24 hr, and the results are given as ±SEM for groups of three at each time point. *P<0.05 **P<0.01. ***P<0.001. *Significant difference from the untreated cells.

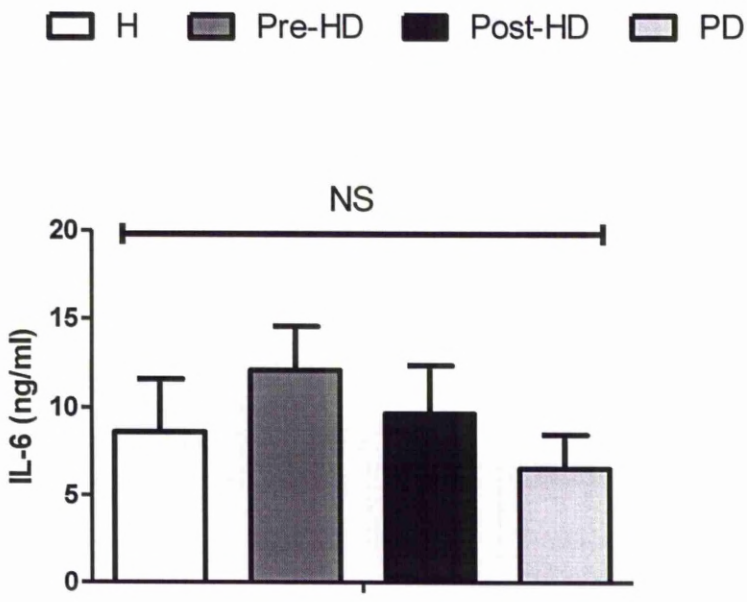

Figure 5.7 Circulating IL-6 levels in human subjects.

Serum concentrations of interlukin-6 (IL-6) in healthy control subjects (white bars), ESRD patients pre-haemodialysis Pre-HD (grey bars), ESRD patients post-haemodialysis Post-HD (black bars) and ESRD patients peritoneal dialysis (light grey bars). Values are means ± SEM ($n = 9$ for control group, $n = 11$ for HD group and $n = 7$ for PD group. NS, not significant.

5.3.6 Serum IL-6 concentrations

Serum IL-6 was measured using ELISA. Serum IL-6 levels in healthy, Pre-HD, Post-HD and PD subjects were 7.63 ± 9.2, 11.11 ± 9.17, 10.12 ± 9.41 ng/mL) and 6.14 ± 5.58 ng/mL respectively (Fig. 5.7). There was no significant difference in serum IL-6 levels among all groups. Due to low sensitivity of used ELISA kit of IL-6 in this study (50 pg/ml), IL-6 levels shoewed higher levels than what been previously reported (Mohamed-Ali et al., 1997)

5.3.7 Measurement of IL-6 gene expression in human adipocytes treated with uraemic serum

The PromoCell adipocytes were used at day 14 post-induction to examine the potential effect of uraemic serum on adipocytes. 0.5 % and 5 % of serum were employed at two time points, 4 and 24 hrs.

The differences in IL-6 mRNA levels among the groups at 4 h with low dose were significant ($p < 0.0001$) and with high dose at 24 h ($p < 0.05$) as IL-6 gene expression was down-regulated 2.5-fold in the pre-HD, post-HD and PD groups (Figure 5.8). *Post hoc* analysis (Bonferroni correction test) showed that the decrease observed for Pre-HD vs H ($p < 0.0001$), Post-HD vs H ($p < 0.0001$) and PD vs H ($p < 0.0001$) and at 24 h was for only Pre-HD vs H ($p < 0.05$) (Figure 5.8).

Comparison of serum treatments from the pre-HD and post-HD indicated that there was no significant change in IL-6 gene expression.

5.3.8 Measurement of MCP-1 gene expression in human adipocytes

The PromoCell adipocytes were used at day 14 post-induction to examine the

potential effect of uraemic serum on adipocytes. 0.5 % and 5 % of serum were employed at two time points, 4 and 24 hrs. At low dose serum and high serum treatment, no significant change was seen in MCP-1 gene expression (Figure 5.9).

Comparison of serum treatments from the pre-HD and post-HD indicated that there was no significant change in MCP-1 gene expression.

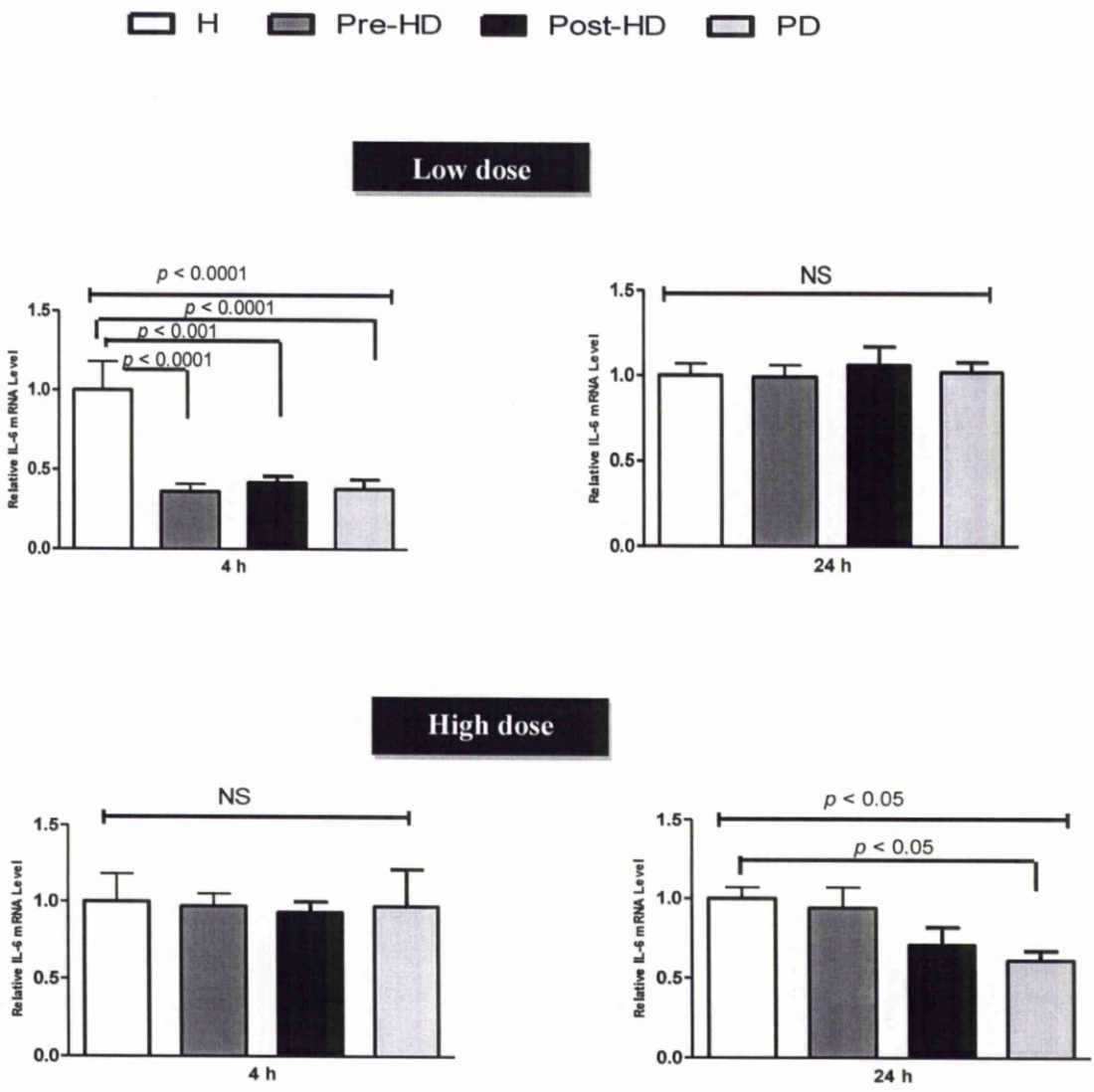

Figure 5.8 IL-6 gene expression levels in adipocytes treated with uraemic serum

Differentiated human adipocytes were exposed to Low dose (upper panel) and to high dose (lower panel) of human pooled sera collected from healthy (H) and ESRD groups pre and post dialysis for 4 and 24 hrs. IL-6 mRNA levels were normalised to human POLR2A and expressed relative to untreated cells. Results

are mean ± SEM (n = 6/group). NS, not significant.

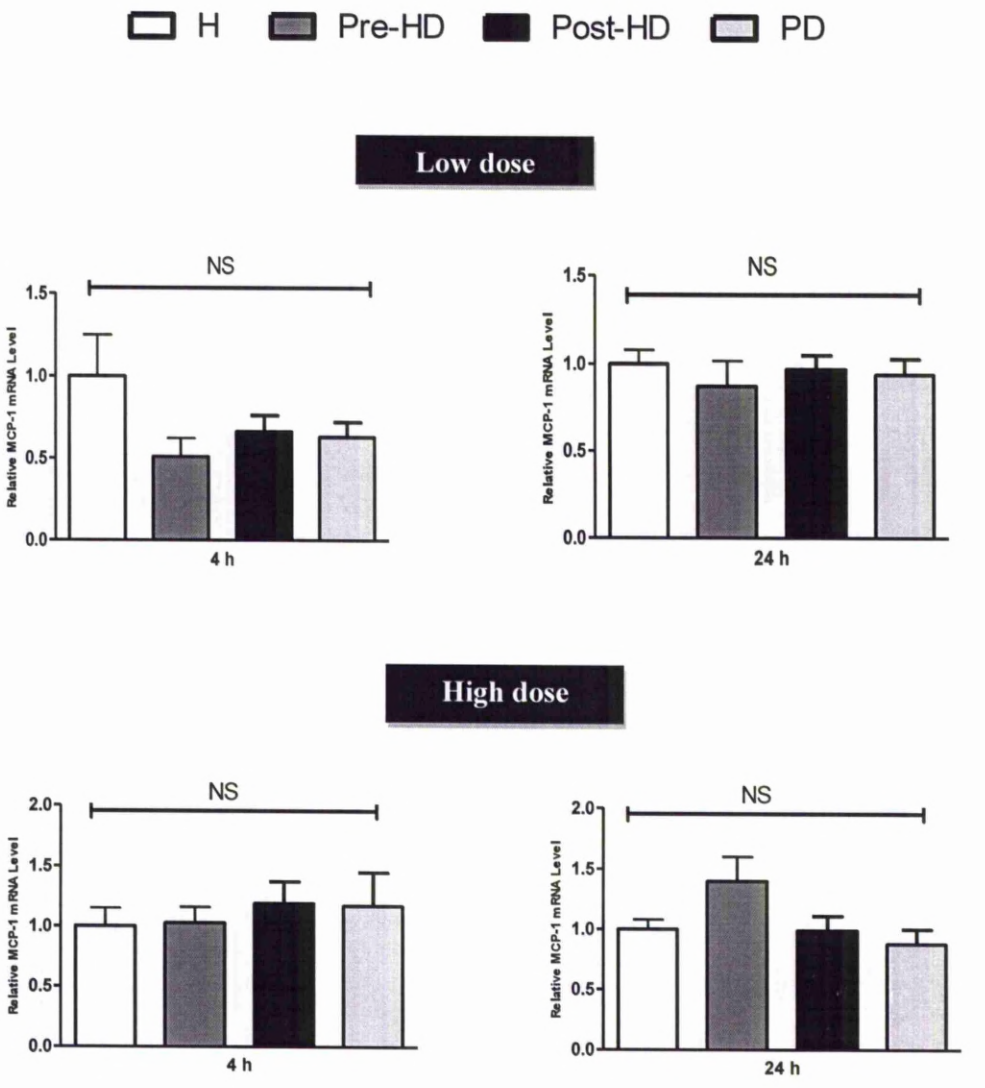

Figure 5.9 MCP-1 gene expression levels in adipocytes treated with uraemic serum

Differentiated human adipocytes were exposed to Low dose (upper panel) and to high dose (lowerpanel) of human pooled sera from healthy (H) and ESRD groups pre and post dialysis for 4 and 24 hrs. MCP-1 mRNA levels were normalised to human POLR2A and expressed relative to untreated cells. Results are mean ±

SEM (n = 6/group). NS, not significant.

5.3.9 Serum leptin concentration

The differences in serum leptin levels among the groups were significant ($p < 0.05$). Serum leptin concentrations in ESRD patients were significantly higher than those in healthy subjects. Haemodialysis group (Pre and Post) presented higher values than peritoneal dialysis groups (57.19 ± 19.57 μg/mL 29.03 ± 12.55 μg/mL; vs 4.80 ± 1.46 μg/mL respectively) (Figure 5.10 A). Figures 5.9 B show the correlation between leptin and body weight in healthy group ($r = 0.2$; $P < 0.14$; $N = 9$) and ESRD group ($r = 0.3$; $P < 0.07$; $N = 18$), respectively.

5.3.10 Measurement of leptin gene expression in human adipocytes

The PromoCell adipocytes were used at day 14 post-induction to examine the potential effect of uraemic serum on adipocytes. 0.5 % and 5 % of serum were employed at two time points, 4 and 24 hrs.

The differences in adiponectin mRNA levels among the groups at 4 h with low dose were significant ($p < 0.05$). At low dose serum for 4 h, leptin gene expression was down-regulated 1.7-fold in PD groups (Figure 5.11 c). However, at 24 h, leptin gene expression was found to be unchanged (Figure 5.11).

Comparison of serum treatments from the pre-HD and post-HD indicated that there was significant increase in leptin gene expression with low dose serum at 24 h.

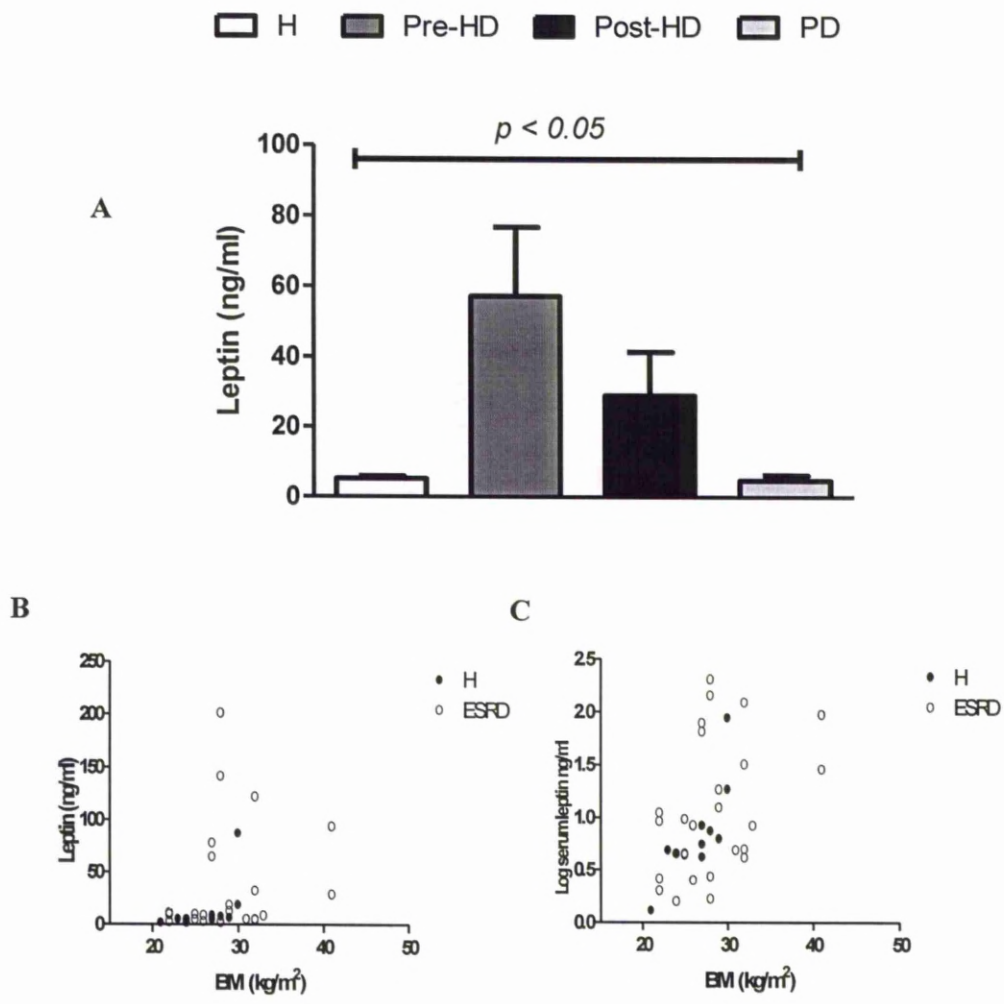

Figure 5.10 Circulating leptin levels in human subjects and BMI correlation

(A) Serum concentrations of Leptin in healthy control subjects (white bars), ESRD patients pre-haemodialysis Pre-HD (grey bars), ESRD patients post-haemodialysis Post-HD (black bars) and ESRD patients peritoneal dialysis (light grey bars). Values are means ± SEM. (B) The correlation between BMI and circulating leptin levels in healthy group (•) ($r = 0.2$; $P < 0.14$; $N = 9$) and ESRD group (○) ($r = 0.3$; $P < 0.07$; $N = 18$), respectively and (C) shows the log of serum leptin plotted against the BMI in the same patients. ($n = 9$ for control group, $n = 11$ for HD group and $n = 7$ for PD group).

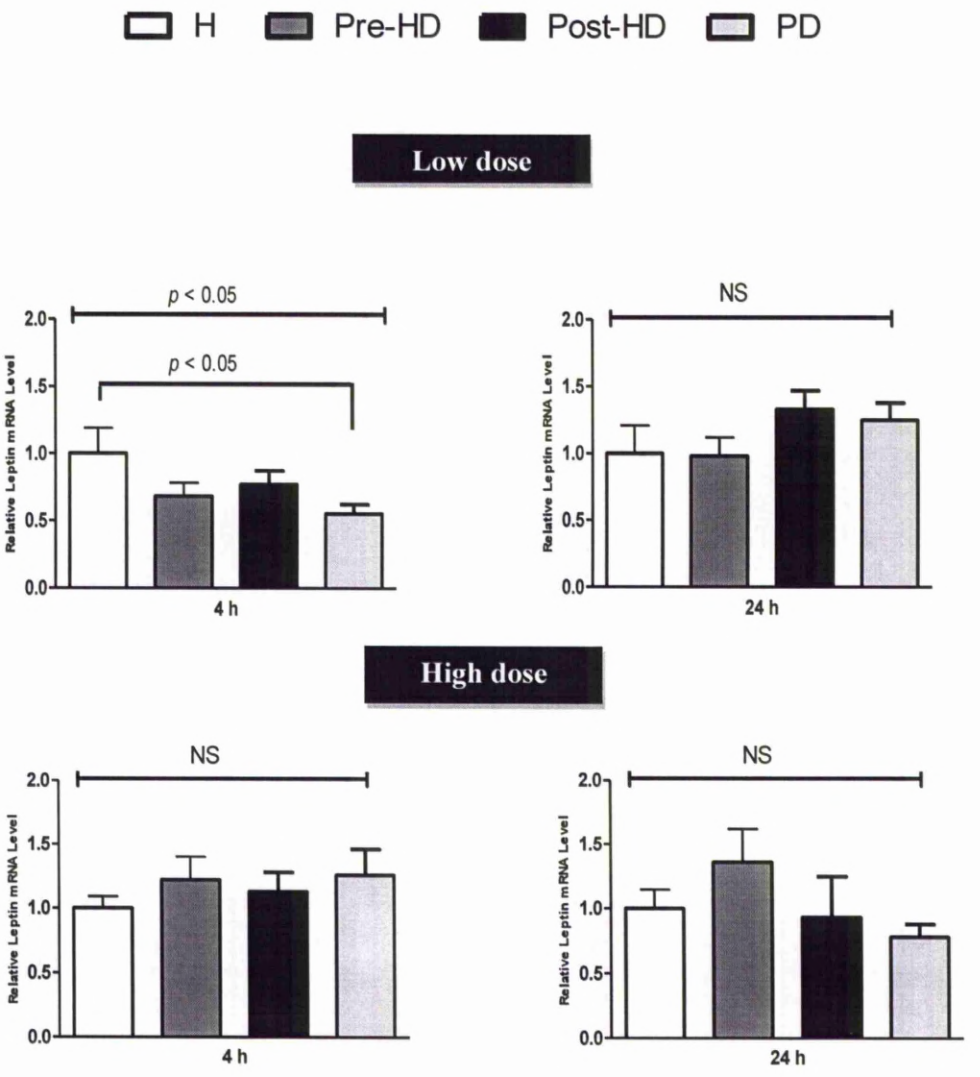

Figure 5.11 Leptin gene expression levels in adipocytes treated with uraemic serum

Differentiated human adipocytes were exposed to Low dose (upper panel) and to high dose (lower panel) of human pooled sera from healthy (H) and ESRD groups pre and post dialysis for 4 and 24 hrs. Leptin mRNA levels were normalised to human POLR2A and expressed relative to untreated cells. Results are mean ± SEM (n = 6/group). NS, not significant.

5.4 Discussion

CVD is considered as the major cause of death in ESRD. However, the mechanism(s) are still not clear but it has been suggested that the uraemic environment could be contributing to this association (Huang et al., 2004). In addition, uraemia-related factors have been shown to play a potential role in the progression of CVD in ESRD patients (Madore, 2003). A role for adipose tissue has been proposed.

The present study, aimed specifically at characterising the role of adipose tissue in the association between CVD and ESRD with attention focused on adipokine expression. The gene expression of key adipokines using an *in vitro* human adipocyte culture system in the presence of serum expected to contain substances and molecules retained in ESRD, collectively termed uraemic toxins, has been investigated.

Initially, plasma collected from healthy subjects and ESRD patients was used in an attempt to standardise the appropriate conditions for cell culture study. For the purpose of analysis, different concentrations of plasma had been separated from pre and post dialysis blood and then incubated for certain period (4 hr and 24 hr) with cultured human adipocytes. The first set of analyses examined the impact of ESRD plasma on biological integrity. The most striking result to emerge from the data was that concentrations more than 15% caused cell death while concentrations less than 10% result in media clotting. As an alternative, serum was tested which was found to be more appropriate for culture studies. Further analysis showed that the optimum serum concentration was between 0.5% and

5%.

The next part of the study, adipnectin expression was investigated in cultured human adipocytes (PromoCell) when exposed to uraemic serum. Adiponectin has favourable properties including antiathergenic, anti-inflammation and insulin sensitizing effects (Costacou and Orchard, 2008). Patients with ESRD undergoing peritoneal dialysis showed a significant increase in adiponectin plasma levels both in total adiponectin and high molecular weight adiponectin compared to the healthy control group. A significant difference in circulating adiponectin levels was seen between Peritoneal and Haemodialysis patients. A similar finding has been reported in previous studies (Bakkaloglu et al., 2005, Huang et al., 2004). Because the adiponectin is a 30 kd molecular weight, it is unlikely to be removed by the dialysis process. However, kidneys are important organs for elimination of many factors including protein hormones; therefore it's likely that impaired kidney function could lead to adiponectin accumulation in the circulation of ESRD patients. Moreover, adiponectin plasma levels were found to be reduced markedly after kidney transplantation but still at higher levels compared to healthy subjects (Chudek et al., 2003). Controversy exists on how the kidneys handle this adipokine (Kataoka and Sharma, 2006).

Uraemia-related factors have been shown to play a potential role in the progression of CVD in ESRD patients (Madore, 2003). Many studies had shown that low levels of adiponectin can be considered as a predictor of incident cardiovascular events in ESRD (Adamczak et al., 2009). Moreover, plasma levels of adiponectin have been shown to be associated negatively with metabolic risk factors (Zoccali et al., 2002). Thus, the higher levels of plasma adiponectin could

be explained by its protective role as anti-atherogenic and insulin sensitizing.

These higher levels of adiponectin in ESRD patients appear not to be from an increase in adipocyte gene expression. The results showed that its gene expression was downregulated significantly in both HD and PD groups with low serum dose. The studies in this thesis are the first to employ normal human adipocytes, exposing these cells to a uraemic environment to show changes in gene expression for proteins/enzymes of lipid metabolism as well as adipokines. This finding is consistent with results from previous studies that indicated that adiponectin gene expression *in vivo* is decreased in patients with ESRD (Marchlewska et al., 2004) as well as in patients with high risk of CVD (Statnick et al., 2000). In one study, there was no difference seen in adiponectin gene expression in peripheral blood mononuclear cells in patients with ESRD (Norata et al., 2009). Moreover, downregulation of adiponectin mRNA has been reported in experimental models *in vitro* as a result of ESRD-associated oxidative stress (Barazzoni et al., 2007). It has been suggested that uraemic toxins might overwhelm the positive effects of adiponectin in the ESRD population (Beige et al., 2009). In the optimization experiment in studies performed in this thesis, adiponectin mRNA was seen to be decreased when serum dose increases, suggesting a dose response. The reasons for the acute and rapid suppression of adiponectin expression and secretion are unknown but may include inflammation and oxidative stress as has been suggested by others. It has been reported that adiponectin gene expression and its release are suppressed by oxidative stress in cultured 3T3-L1 adipocytes (Furukawa et al., 2004). Moreover, low gene expression has been associated with oxidative stress in non-diabetic ESRD patients following adipose tissue biopsy

studies (Barazzoni et al., 2007). In addition, adiponectinaemia has been found to lower gene expression by a feedback mechanism (Bauche et al., 2006, Marchlewska et al., 2004). Since it has been suggested that secretion of adiponectin is under feedback regulation, it could be argued that since adiponectin was increased in the uraemic serum that was added to the adipocyte *in vitro* in the studies described in this thesis, this may have resulted in an autoregulatory inhibition of adiponectin transcription and secretion. Receptors for adiponectin are present on adipocytes. This argument would also be consistent with a dose effect of the uraemic serum seen in our studies. This hypothesis could be refuted by direct addition of adiponectin to the adipocyte cultures, not performed in these studies. Also, healthy serum *per se* downregulated adiponectin expression in adipocytes suggesting a response to unknown humoral components found in serum (Körner et al., 2005). It is possible that increased circulating adipokines, other than adiponectin, such as IL-6 and leptin found in this study and others not measured here, could influence expression and secretion of adiponectin.

Leptin is one of the most intensively studied adipokines. Leptin is mainly synthesised in adipocytes and its plasma levels are proportional to adiposity. Leptin is involved in food intake regulation as well as other functions. It has been documented that deficiency in leptin levels results in diabetes and obesity as a consequence of high food intake (Kataoka and Sharma, 2006). Similar to previous studies, the results presented in this thesis show that patients prior to undergoing haemodialysis have a significant change of circulating levels of leptin when among the studied group. Hyperleptinaemia has been reported to be associated with ESRD (Fontán et al., 1999, Sharma et al., 1997). However, these higher

levels in haemodialysis is probably not caused by high expression as the results have shown a little changes in adipocyte leptin gene expression when treated with ureamic serum. If anything, supporting a trend of downregulation of leptin expression when pre- and post-HD sera were added, only reaching significance in the peritoneal group, there was a significant downregulation of leptin mRNA levels and that could be a possible explanation for non-increase in the circulating levels with this group. It has been also documented that leptin gene expression is decreased in patients with ESRD (Nordfors et al., 1998). Collectively, like for adiponectin, autoregulation of leptin expression and secretion by leptin itself cannot be excluded. As with adiponectin modulation of adipokines secretion by other adipokines cannot be excluded. Leptin is strongly linked with nutrition and may have similar implications in the studies presented in this thesis.

Data from the present thesis showed that serum leptin levels did not associated with BMI. Although ESRD prevents elimination of leptin and leads to high circulating concentrations (Merabet et al., 1997), BMI values of ESRD were similar to the healthy group in this thesis. However, some studies failed to show a positive association between serum leptin levels and nutritional status markers such as BMI and fat mass (Beberashvili et al., 2011) similar to those in this thesis. Moreover, kidney transplantation resulted in normal levels of serum leptin confirming an important role for the kidney in the elimination of circulating leptin (Kokot et al., 1998). Although there are limited studies showing the role of adipokines in renal pathology, leptin has been proposed to be involved in this process (Carrero et al., 2010). The higher levels of serum leptin have been suggested to be involved in the pathogenesis and progression of obesity-related

kidney disease (Kambham et al., 2001). It has also been shown that infusion of leptin in normal rat resulted in development of glomerulosclerosis and proteinuria (Wolf et al., 1999). The significance of increased circulating leptin in progression of earlier stages of renal failure may be important but its significance in ESRD is unclear .

It is well-known that ESRD is associated with chronic inflammation, oxidative stress and endothelial dysfunction (Stenvinkel, 2006). Therefore, two proinflammatory markers, IL-6 and MCP-1 gene expression were investigated. The results have shown that there is a trend towards increased in circulating levels of IL-6 in Haemodialysis groups which was not statistically significant. However, a number of studies have reported that IL-6 is not elevated in all ESRD patients (Stenvinkel et al., 2005). The higher levels of IL-6 are considered a strong predictor for cardiovascular disease in ESRD (Stenvinkel et al., 2002b). Moreover, the higher levels of IL-6 in ESRD patients have been attributed to the long half life of IL-6 along the presence of chronic inflammation (Jacobs et al., 2004). The differences of IL-6 gene expression were observed in all patents group at low dose. IL-6 studied in this thesis related to the adipocyte contribution. Clearly inflammatory cells such as the monocyte/macrophage system may contribute to the inflammatory mileau, and were not studied in this thesis.

MCP-1 has been proposed to play an important role in many disease states such as inflammation, atherosclerosis, diabetes and nephropathies (Yadav et al., 2010). The results have shown that there was no significant in MCP-1 adipocyte gene expression. Circulating MCP protein was not measured in this thesis and an increase in concentration cannot be excluded. Post-transcriptional regulation can

lead to increased production and secretion of cellular proteins. Therefore, we cannot exclude the possibility of altered circulating MCP-1 protein.

Several investigations have been reported that adipose tissue is involved in ESRD. It has also been reported that a low BMI can be an important predictor for mortality in the haemodialysis population (Kalantar-Zadeh et al., 2006). In contrast, other reports have been showed that high BMI is associated with better survival (Friedman, 2006, Honda et al., 2007). Moreover, the change in fat mass over time has been considered as a risk factor for mortality in haemodialysis patients (Kalantar-Zadeh et al., 2006).

In peritoneal dialysis, patents tend to gain more weight due to glucose-containing peritoneal dialysis solution. This increase in glucose may lead to increase lipogenesis in the liver and this can be considered as a potential risk factor for the development of CVD in ESRD in the peritoneal dialysis population (Fernstrom et al., 1998, Klim and Williamson, 1983).

There is controversy as to the source of adiponectin from adipose tissue, whether it is mainly from subcutaneous (Lihn et al., 2004) or alternatively from the visceral depot (Degawa-Yamauchi et al., 2005). Cultured adipocytes in this study originated from the subcutaneous depot. Different factors such as differentiation capacity, cell viability, serum-containing media and many others might play a role in the expression of adipokines. .

Gene expression at the mRNA level is generally informative but not predictive for that at the protein level. Thus, further investigation for protein detection may allow for a deeper interpretation.

In conclusion, an *in vitro* system to study the effect of uraemic serum on adipose tissue function through adipocyte gene expression was established. The results from the modulation of this *in vitro* adipocyte assay system not only serves as a convenient and unique model to study mechanisms of disease such as ESRD and its treatments but has so far also provided an initial insight in understanding the putative role of adipose tissue contributing to the associated of CVD risk in ESRD patients. Data also suggests that normal adipocyte function is altered when exposed to a uraemic environment *in vitro*.

CHAPTER 6

ADIPOSE TISSUE AND INFLAMMATION IN END-STAGE RENAL DISEASE

6.1 Introduction

In addition to the important metabolic function, adipose tissue is considered a very active endocrine organ which secretes numerous hormones and adipokines that are linked to inflammation, insulin resistance and endothelial dysfunction (Chudek and Wiecek, 2006, Wellen and Hotamisligil, 2005). Adipose tissue is a heterogeneous tissue, which consists of many cell types in addition to adipocytes including preadipocytes, macrophages and vascular cells. An increase in adipose tissue mass is accompanied by an increased adipocyte size and number that may have many pathophysiological consequences. In altered adipose tissue states such as obesity, the production of pro-inflammatory adipokines has been linked to many metabolic complications (Weisberg et al., 2003). The expression and production of proinflammatory adipokines, such as tumor necrosis factor α (TNFα), interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1), and tissue infiltration of adipose tissue by inflammatory cells have been shown to be increased in obese and insulin-resistant subjects. (de Luca and Olefsky, 2008).

It has been reported that obesity and insulin resistance are associated with macrophage accumulation in adipose tissue (Weisberg et al., 2003, Xu et al., 2003). Furthermore, increased production of pro-inflammatory adipokines from adipose tissue may contribute to the CVD and malnutrition in ESRD (Stenvinkel, 2006). However, it has been recently accepted that adipocytes are not the main producer of inflammatory mediators secreted from adipose tissue. Adipocyte mass is associated with BMI and insulin resistance and also with macrophage infiltration (Weisberg et al., 2003). Macrophages in adipose tissue (MAT) have been proposed as the main producer for inflammatory mediators rather than

adipocytes (Surmi and Hasty, 2008). Patients with ESRD have a significantly high rate of morbidity and mortality from CVD . Inflammation in ESRD has been suggested to contribute to the CVD progression (Silverstein, 2009, Stenvinkel, 2006). The present study was performed to investigate the extent of macrophage infiltration into adipose tissue in ESRD and to compare ESRD with non-ESRD human subjects.

6.2 Methods

6.2.1 Patients adipose tissue

Six male patients with ESRD (mean age 52±16.2 y, BMI 26.4±2.4 kg/m^2) who underwent kidney transplantation were included in the study. Causes of ESRD were chronic glomerulonephritis, diabetic nephropathy and polycystic kidney disease. Patients on haemodialysis therapy received 3 to 4 hours of therapy 3 times per week. The majority of patients were administered antihypertensive medications (angiotensin-converting enzyme inhibitors and/or angiotensin II receptor antagonists, β-blockers, calcium channel blockers) and other drugs commonly used in patients with ESRD, such as phosphate and potassium binders, diuretics, erythropoiesis-stimulating agents, iron substitution, and vitamin B, C, and D supplements.

The study protocol was approved by Liverpool Research Ethics Committee (REC number: 08/H1002/41), and fully informed and written consent was obtained in all cases.

6.2.2 Non-ESRD adipose tissue

The non-ESRD group consisted of 6 men with no ESRD or any medication (mean

age 61.2±18.5 y, BMI 26±2.6 kg/m^2) who underwent elective cholecystectomy or kidney donation at Royal Liverpool University Hospital. Fat biopsies were obtained from two different locations; subcutaneous (n = 6), and omental (n = 6). Fully informed and written consent was obtained in all cases.

6. 2.2 Immunohistochemistry for CD-68 in adipose tissue

CD-68 staining was used as a marker for macrophages. Staining was performed using a standard protocol on sections from formalin-fixed paraffin-embedded tissue blocks. Five-micron thick sections were microwave-treated in 10 mmol/l citrate buffer (pH 6.0) and incubated for 1 hour at room temperature with primary antibodies; mouse monoclonal antibody CD-68 clones KPI (Dako Cytomation). After rinsing in PBS buffer containing 0.25 % Triton X-100 (pH 7.2), sections were incubated with secondary biotinylated goat anti-mouse (Vector). Avidin-biotin peroxidase complexes (Vector) were added followed by visualization with 3.3′-diaminobenzidine tetrachloride (Vector). All sections were counterstained with Harris haematoxylin. Tissue sections incubated without primary antibody were used as negative controls. Tissue sections incubated without primary antibody were used as negative controls.

6.2.3 IHC scoring

For each subject, the number of macrophages (identified as CD-68-positive cells) within the entire section were counted using a light microscope (Polyvar microscope - Leica). The percentage of positively stained cells was scored semiquantitatively in a "blinded" manner. The number of CD68-positive cells per high-power field was counted.

6.2.4 Statistical analysis

Data are expressed as means ± SD. Differences between study groups in tissue staining score were tested using Mann-Whitney U test. Differences were considered as statistically significant when $P < 0.05$.

6.3 Results

Adipose tissues from 6 ESRD patients undergoing kidney transplantation (12 samples) were procured for this study and the same number also from non ESRD subjects with no differences in BMI or sex distribution (Table 6.1). Positive staining for the macrophage marker CD-68 was investigated in 24 adipose tissue samples. Two different depots (subcutaneous and omental) were examined from participating subjects. The total number of macrophages per section area in subcutaneous adipose tissues did not differ between the groups, despite a tendency toward higher cell number in the ESRD group (Figure 6.1). The total number of macrophages per section area did not differ between the groups, despite a tendency toward higher cell number in the ESRD group in subcutaneous and omental depots (2.8±0.75 vs.1.8±0.5 and 3±0.0 vs.1.3±0.6, respectively, NS) .

	Non-ESRD	ESRD
n	6	6
Age (years)	61.2(18.5)	52(16.2)
BMI(kg/m$^{2)}$	26(2.6)	26.4(2.4)

Table 6.1 Clinical characteristics of patients

Data are expressed as Mean ±SD

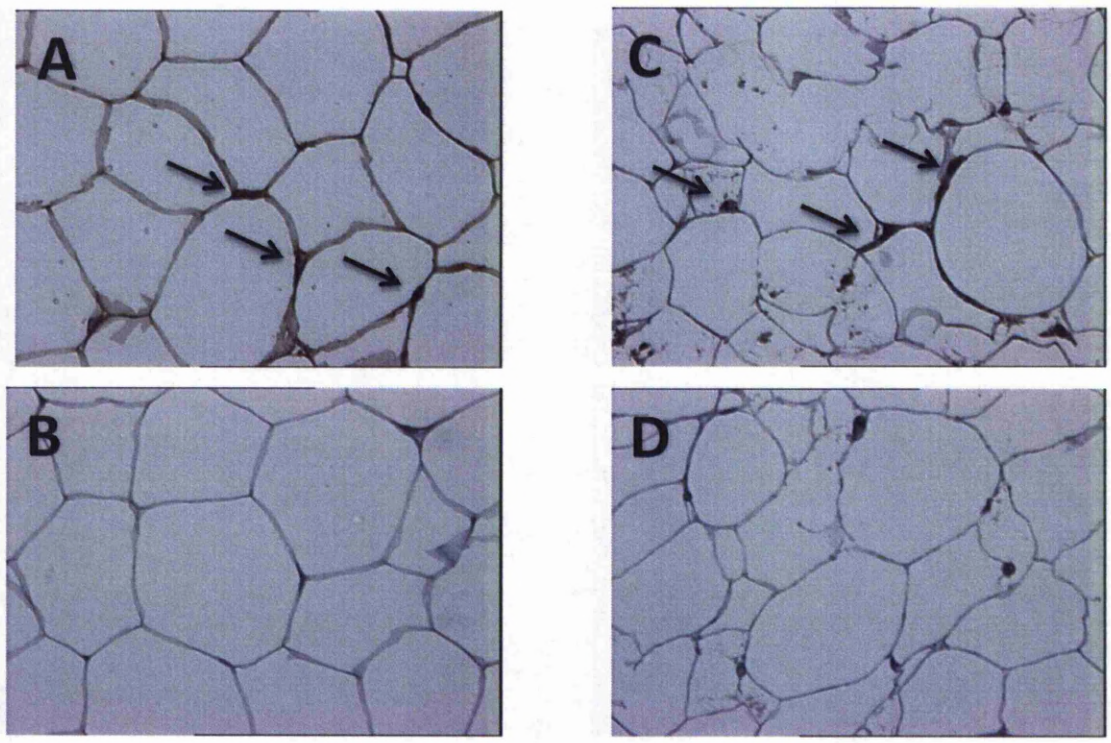

Figure 6.1: Immunohistochemical detection of CD-68 macrophages in human subcutaneous adipose tissue.

Subcutaneous adipose tissue samples were taken from the subcutaneous abdominal region of human healthy non-ESRD subject whose BMI 30 kg/m^2 (A and B) and ESRD subject whose BMI 30.2 kg/m^2 (C and D). B and D represent negative control not stained with CD-68 antibody. Arrows are macrophages. Magnification x40

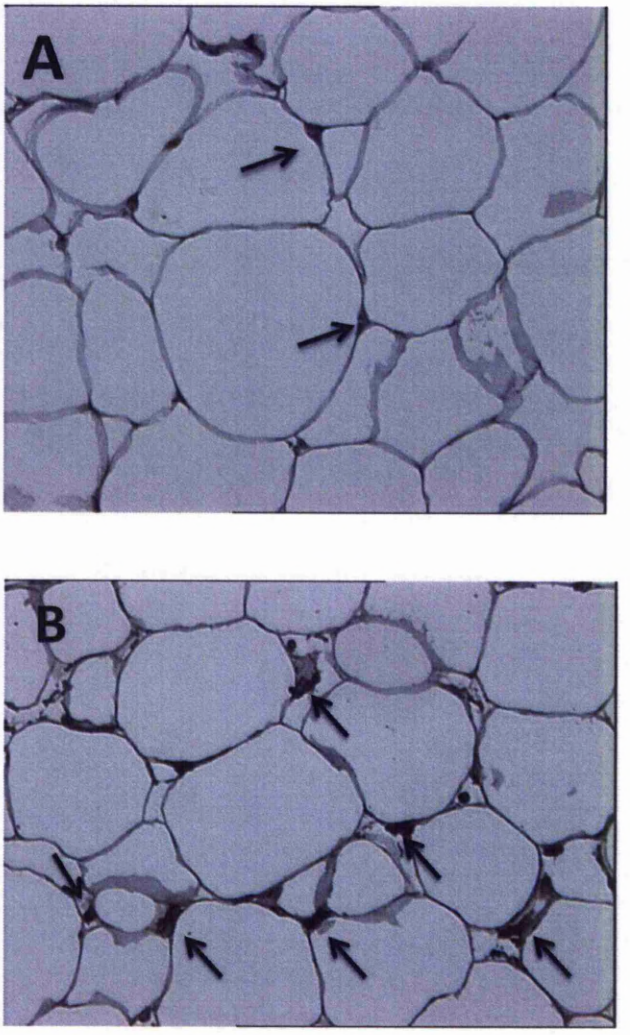

Figure 6.3: Immunohistochemical detection of CD-68 macrophages in human omental adipose tissue.

Visceral adipose tissue samples were taken from the omental region of human healthy non-ESRD subject whose BMI 30 kg/m^2 (A) and ESRD subject whose BMI 30.2 kg/m^2 (B). Arrows are macrophages. Magnification x40

6.4 Discussion

In the present study, macrophage infiltration into adipose tissue was investigated in ESRD patients in different depots. The main finding was that adipose tissue from ESRD tended to have higher number of macrophage cells but did not achieve statistical significance.

It has been reported that proinflammatory markers are considered as strong predictors of mortality of CVD in ESRD (Axelsson et al., 2004). Inflammation is a common feature of ESRD and is associated with an adverse outcome (Stenvinkel et al., 2004). Moreover, it has been suggested that ESRD has a common feature namely the metabolic syndrome of obesity (Beddhu et al., 2005). Furthermore, it has been reported that ESRD is associated with metabolic syndrome-related complication such as inflammation, dyslipidaemia and atherosclerosis (Zanetti et al., 2008). The high prevalence of ESRD can be attributed to many causes including obesity (Kalaitzidis and Siamopoulos, 2011). Obesity has many implications for the dialysis patients (Zoccali and Mallamaci, 2011). Obesity is now considered as a chronic and low-grade inflammation state (Trayhurn, 2005). Obesity is characterised by hyperplasia and hypertrophy of adipocytes during the accumulation of fat mass and is associated with macrophage infiltration (Torres-Leal et al., 2010).

Gene expression of adipocytes was found to be similar as in macrophage and these types of cells share functional capabilities (Charrière et al., 2003). Moreover, there been an accumulation of evidence that adipose tissue has a link with immune system in terms of energy metabolism (Schäffler et al., 2006).

Fat mass has been found to be correlated with high level of circulating

proinflammatory markers in ESRD (Axelsson et al., 2004). However, it has been explained that increase expression of inflammatory marker can be derived from macrophage rather than adipocytes. Xu et al (Xu et al., 2003) and Weisberg et al (Weisberg et al., 2003) published the first report that obesity is associated with macrophage infiltration. In these two studies, macrophages are suggested to be main contributors to the inflammatory mediator production from the adipose tissues studied. Macrophage marker CD-68 expression was found to be significantly higher in adipose tissue of obese compared to lean subjects in these previous pioneering observational studies.

Recently, two studies have been published to investigate CD-68 in ESRD patients. Roubicek et al found an increase macrophage infiltration of subcutaneous and visceral adipose tissue in women with ESRD (Roubicek et al., 2009). However, Witasp et al found no difference in CD-68 expression between ESRD and control (Witasp et al., 2010). The latter finding is consistent with those of the present study as the results showed no significant difference in the number of CD-68 between studied groups. Moreover, it has been reported that fat mass is associated with increased CD-163, a circulating marker of macrophages (Axelsson et al., 2006b), a measurement that was not carried out in the present studies.

The mechanisms for macrophage infiltration in adipose tissue remains not fully understood. However, two mechanisms have been proposed by which macrophages can infiltrate the adipose tissue. First, macrophage can be originate from bone marrow-derived monocytes; second, macrophages can trans-differentiate from preadipocytes or mesenchymal stem cells within the adipose tissue itself (Schäffler et al., 2006). However, it has been noted that fat mass is

strong predictor of macrophage infiltration into adipose tissue (Tchoukalova et al., 2007). There are many factors have that been suggested to play a potential role in macrophage recruitment and infiltration including adipocyte hyperplasia, adipocyte hypertrophy, altered adipokine production, apoptosis and hypoxia (Bourlier and Bouloumie, 2009). Hypertrophic adipocytes have been showed to have a high proinflammatory adipokine profile compared to smaller cells (Skurk et al., 2007). Furthermore, macrophage infiltration into adipose tissue has been suggested to play role in insulin resistance and systemic inflammation (Bouloumie et al., 2005).

Adipose tissue secretes hundreds of biologically active molecules that influence many physiological processes. It is therefore no surprise when thirty percent of these molecules are inflammatory and macrophage-specific(Schäffler et al., 2006). Although there is similarity between adipose tissue functions in ESRD and obesity, there were also significant differences. One of the major differences is that adiponectin, an adipose tissue-derived hormone with anti-inflammatory, anti-atherogenic and insulin sensitizing properties is found at high levels in the circulation of ESRD compared to obese subjects. Moreover, adipose tissue may contribute to systemic inflammation in ESRD (Barazzoni et al., 2011). It has been shown that systemic inflammation may be associated with cardiovascular events (Berg and Scherer, 2005).

In conclusion, the present study shows a tendency towards an increased accumulation of macrophages in adipose tissue in ESRD patients. Increasing the statistical power of such studies either by collecting adipose tissue samples from more subjects or by studying more sections and thereby counting more cells in

more fields may be necessary. We are considering whether we could increase our statistical power by counting more sections from tissues already collected. It is necessary to bear in mind that adipose tissue and other samples collected in this and previous studies described in this thesis came from intensively treated patients both by pharmacological measures such as drugs as well as dialysis, thereby perhaps minimising the abnormal pathophysiology. One possibility is to study animal models of renal failure not confounded by treatment effects. Time constraints prevented collection of more samples. Despite these limitations, it is likely that inflammation of the adipose tissue in ESRD is a reality, but requires more proof. It remains to be determined how important this adipose tissue-mediated inflammation is in contributing to the systemic proinflammatory state in uraemia, to cardiovascular disease and to progression of renal dysfunction in these patients and whether interventions suppressing such inflammation may have beneficial effects.

CHAPTER 7

GENERAL DISCUSSION

General introductory comments

The hypothesis that has driven this thesis was based on the knowledge that adipose tissue, which has been shown to contribute to CVD in various ways that include alteration in lipoprotein metabolism as well as secretion of pro and anti-inflammatory adipokines in states without ESRD, may also be involved in ESRD. The proposal is that the retention of uraemic toxins in the body of ESRD patients may lead to adipose tissue dysfunction affecting both adipokine production and lipoprotein metabolism – a potential link to CVD. The specific aim was to isolate and characterize the role of adipose tissue in ESRD in terms of lipoprotein metabolism and adipokine expression and secretion.

Rationale for studies

The rationale for studies in this thesis was that the retained substances in ESRD could alter adipose tissue function. This was tested in two ways. One was to obtain adipose tissue from ESRD patients undergoing renal transplantation. This would provide information about the effect on long-term exposure to uraemic environment. Although we would have liked to have measured expression of adipokines and lipid metabolism genes in adipose tissue biopsies, time constraints prevented this. The second way we tested the possible alteration of adipose tissue by uraemic environment was to expose normal adipocytes to serum from ESRD patients to see if the lipid metabolism or adipokine function is altered in any way. These changes are clearly different from the effects of uraemic environment in ESRD patients since the adipose tissue in ESRD would have been exposed to

uraemic substances chronically. Nevertheless changes were observed both in adipose tissue biopsy samples as well as the *in vitro* adipocyte culture studies.

Focus on aspects of lipoprotein and adipokine metabolism in chapters

The main outcomes presented in this thesis focused on the effect of uraemic milieu on adipose tissue function. The results presented in chapter 3 to 6 have shown that both uraemic serum and uraemic lipoproteins altered adipokine expression, particularly those with favourable properties such as adiponectin. The results presented in chapter 3 corresponded to observations regarding lipoprotein profile in ESRD patients who were undergoing either haemodialysis or peritoneal dialysis. Chapter 5 was dedicated to adipokine gene expression of adiponectin, leptin, IL-6 and MCP-1 in human adipocytes culture in the light of uraemic environment. Chapter 6 relates to inflammation in adipose tissue by detecting of macrophage marker CD-68. Chapter 4 focused on the gene expression of lipid metabolism and included LPL, HSL, LDL-R and C3 in the human adipocytes culture exposed to the different uraemic lipoproteins.

Analytical techniques employed

The techniques utilised in all the studies of human adipocytes culture include real-time PCR for adipokine and lipid metabolism related factor gene expression, density ultracentrifugation for VLDL, LDL and HDL fractionation, ELISA for the analysis of protein secretion and plasma concentration and immunohistochemistry for detection of CD-68 expression in adipose tissue. During this study, other techniques were also employed, ELISA and standard PCR.

Interaction between adipose tissue and lipoproteins

Adipose tissue has a crucial role in storing nutrients predominantly dietary triglycerides following intake in the postprandial state. This involves sequentially hydrolysis of chylomicron (and indirectly VLDL) by lipoprotein lipase, release of fatty acids and uptake of fatty acids into adipocytes prior to re-esterification and storage. Lipoprotein lipase is therefore a key gatekeeper for the storage of dietary triglycerides. The present thesis showed that the nutrient storage function could be compromised in ESRD in several ways. Although direct measurement of LPL was not performed, gene expression for LPL showed significant decrease when normal adipocytes were exposed to uraemic pooled sera; this would be consistent with a decrease in LPL protein and activity and supports similar reported results by other groups (Dautin et al., 2007). Circulating triglycerides in serum and VLDL lipoproteins were also significantly increased compared with healthy sera, both consistent with decreased clearance of triglyceride-rich lipoproteins in the uraemia of ESRD. Similar findings of hypertriglyceridaemia in ESRD have been reported by other groups (Tsimihodimos et al., 2011, van de Woestijne et al., 2011). These findings are strongly suggestive of a defective adipose tissue storage function in ESRD.

On the other hand appropriate release of fatty acids from stores is required in the post-absorptive and prolonged fasting states. This fatty acid release is necessary to allow cells, tissues and organs that can utilise fatty acids for energy purposes to survive during periods of energy deprivation. The key enzyme that is classically involved in hydrolysing triglycerides droplets from adipose tissue stores is hormone-sensitive lipase. The data in this thesis suggest that normal adipocytes

when exposed to the uraemic sera down-regulate expression of hormone sensitive lipase genes; this is also consistent with the idea that release of fatty acids could be impaired in uraemia although confirmation will require protein measurements of HSL. Unfortunately circulating free fatty acid measurements were not carried out in the sera to further support these observations.

It could therefore be argued that the adipose tissue in ESRD could be characterised by an inability to take up nutrients and also an inability to release these nutrients when required. This would suggest an abnormality in a fundamental property of white adipose tissue suggesting that it may be dysfunctional in ESRD.

Besides triglyceride storage, dietary (and circulating) cholesterol is also taken up by white adipose tissue. Therefore in addition to circulating lipid and lipoprotein measurements, LDL-receptor gene expression was examined in this thesis. Despite down regulation of gene expression of LDL-R in this thesis, no differences in total and LDL-cholesterol in HD and PD groups compared with normal, were seen. One possible explanation for this could be that LDL could be cleared by other mechanisms including through the scavenger receptor on macrophages, a potentially atherogenic process.

It could therefore be argued that the 'dysfunctional' adipocytes in uraemic states could contribute to defective energy metabolism through impairment of triglyceride metabolism and also contribute to atherogenesis through defective cholesterol uptake. Other investigators have also observed that uraemia by down regulating VLDL receptor in adipose tissues can limit the long-term storage of

energy and can contribute, in part, to wasting and weight loss in ESRD (Vaziri, 2006).

Effect of dialysis

The cardiovascular risk remains very high in ESRD despite haemodialysis and peritoneal dialysis. Hyperlipidaemia is a well established risk factor of cardiovascular disease and it is therefore of interest to determine if HD affects circulating lipids.

The result in this thesis showed that haemodialysis may have effects on plasma lipids. This effect of haemodialysis includes increase in plasma total cholesterol and HDL-cholesterol. However, small increases in TG and LDL-cholesterol levels were also seen but were not statistically significant. Similar findings has been observed and attributed to the consequence of acetate used instead of bicarbonate as a dialysate buffer (Yalçin et al., 1999). Although mild to moderate increases in apolipoproteins (not measured in this thesis) have been reported following the institution of haemodialysis (Mesquita et al., 2010) , lipoprotein fractions did not show differences in this study between HD and PD. This is contrast to other data which suggested that treatment with either HD or PD may modulate plasma lipoprotein values (Attman et al., 2003). Overall, the effect of haemodialysis on lipid/lipoprotein abnormalities in ESRD does not appear to be major.

Dialysis is an important component of managing ESRD. Traditionally this has employed peritoneal or haemodialysis. Haemodialysis is used more widely and is excellent at clearing molecules less than 500 daltons. It has long been recognised that molecules between 500 to 5000 daltons, called middle molecules, are pathophysiologically important in uraemic syndrome. Conventional haemodialysis

is poor at clearing the 'middle molecules'. A new innovation, haemodiafiltration, is more effective at clearing the middle molecules (Vanholder et al., 2008a). Since the cardiovascular mortality is very high in patients on haemodialysis, there is interest in determining whether post-dialysis metabolic mileau is less or more atherogenic.

Although many studies have reported that peritoneal dialysis is associated more with dyslipidaemia rather than haemodialysis (Attman et al., 2003), data obtained in this study was unable to detect any significant difference in lipid profile compared to healthy control. PD caused mild increases in plasma total cholesterol, LDL-cholesterol and VLDL-cholesterol in this thesis. Plasma HDL-cholesterol levels were significantly higher in PD patients compared to Pre-HD patients and surprisingly PD patients had normal levels of plasma TG. It is unlikely that this difference between HD and PD can be attributed to the nutritional status as there was no difference in BMI. However, the non-change in lipid profile could be explained by the low number of recruited subjects in this group and also that all patients were unavoidably under lipid lowering medication, such as statin.

When we started these studies, we were not aware of any other data that could have guided us. Because the studies in this thesis required detailed metabolic characterization, we believed that the numbers recruited would be sufficient in this pilot project. In retroscpect, we agree that studying a greater number of patients would have yielded better results. However, this was beyond the scope of the studies and will clearly need to be done future follow-up studies. Despite this, athough the numbers were small in these studies, the dialysis subjects showed an effect on the adipose tissue function in this pilot study. The preliminary results

indicate that ESRD had altered lipid metabolism by downregulate lipolytic enzymes as well as by altering the lipid phenotype into an atherogenic lipid profile. Moreover, it is evident that changes in adipokines and enzymes studied were reliable and significant in some cases. Results in this study have shed some light on ESRD and its relationship with adipose tissue, where previously such knowledge was limited. Clearly, it will be necessary to conduct further studies to confirm the results of this thesis and extend these further involving a greater number of subjects.

The results in this thesis found no changes in the measurements made in before and after HD, suggesting that HD did not modify the 'risk' factors measured in this thesis. One interpretation of this would be that this would support the high CVD mortality despite HD. This does not exclude the possibility that other solutes not measured in this thesis could be playing a significant part in the CVD risk of ESRD. ESRD has been prioritised for early detection to allow early intervention (Winkelmayer et al., 2001). This is because ESRD is associated with marked increase in cardiovascular morbidity and mortality (Schoenhagen and Tuzcu, 2002). Staging of CKD, done by an estimation of GFR employing validated equations using plasma creatinine, is now routinely performed. Five stages (1 to 5) of CKD have been described using this approach with CKD 5 being end-stage renal failure. It has also become clear than less than 10% of people with CKD enter the end stage (ESRD), the majority dying from CVD earlier in the natural history of CKD (Wizemann, 1998). Traditional risk factors for CVD do not explain this increase in morbidity and mortality in ESRD. There is an urgent need to understand the reasons for this increased risk.

ESRD patients may be malnourished or obese and the issue of nutrition in prognosis of ESRD is still under debate (Kaysen, 2006b, Stenvinkel et al., 2002a). Increased visceral obesity is also a feature of ESRD. Metabolic syndrome characterised by increased visceral adipose tissue is now known to be causally related to CVD. The studies in this thesis except for those in Chapter 6 were mainly on subcutaneous adipose tissue largely due to practical difficulties in carrying out such studies on visceral fat. Alternative approaches are necessary to understand the contribution of the visceral adipose tissue in ESRD to CVD.

Lipoproteins in ESRD

Lipoproteins are central to the pathogenesis of atherosclerosis and CVD and these include LDL, HDL, VLDL and chylomicrons. Lipoprotein metabolism is altered in ESRD and its full characterisation may be important to clarify CVD in ESRD. Adipose tissue alters plasma lipoproteins both in health, obesity and anorexia (Zhou et al., 2006). There is evidence that lipoproteins interact with the adipose tissue not just in order to engage in the nutrient functions but also in terms of adipokine secretion (Huang et al., 2009, Zhou et al., 2006). Adipocytes phagocytose lipoproteins and are involved in their clearance. Lipoproteins are exposed to a uraemic environment in ESRD and thus may be qualitatively and quantitatively altered. Little is known about the interaction between the lipoproteins and adipose tissue in ESRD.

The present study demonstrates that ESRD patients had altered the lipoprotein profile. Major lipoprotein fractions VLDL, LDL and HDL were separated successfully by apply iodixanol gradient ultracentrifugation. The aim from

fractionation was first to explore and characterize any lipoprotein abnormalities in ESRD in terms of haemodialysis and peritoneal dialysis and second, to incubate these fractions with human adipocyte culture in order to examine their potential effect on lipid metabolism-related factor expression, achieved in chapter 4. Iodixanol gradient ultracentrifugation is a convenient, rapid and reliable method for lipoprotein fraction separation (Graham et al., 1996). Cholesterol distribution of lipoprotein fractions showed a clear abnormal profile with VLDL enrichment, shift in peak LDL towards IDL and presence of small dense LDL. Although plasma levels of HDL-cholesterol were low in ESRD patients, HDL-cholesterol distribution failed to confirm this possibly because of a contamination with plasma protein especially albumin and this one disadvantage of using the gradient ultracentrifugation technique (Yee et al., 2008). Despite this a change in structure of HDL with smaller particles predominating and also showing increased proportion of cholesterol compared with normal controls was seen.

VLDL-cholesterol, LDL-cholesterol and IDL-cholesterol have been reported as independent risk factors for atherosclerosis in ESRD patients (Shoji et al., 2001). Several mechanisms have been proposed to explain lipoprotein abnormalities in ESRD, mentioned in chapters 3 and 4. The main lipoprotein abnormality that was identified was a decrease in plasma clearance of triglyceride-rich lipoproteins and decrease in plasma levels of HDL. Although earlier studies had suggested that dyslipidaemia was involved in the renal disease progression, mechanisms responsible for this are still unclear (Crook et al., 2003). In addition, LDL and apo-B have been demonstrated to be associated with significant renal dysfunction (Samuelsson et al., 1997). The mechanism of remodeling of lipoproteins when

circulating triglycerides are increased results in the atherogenic lipoprotein phenotype that has been well researched and understood (Kaysen, 2011). This remodelling results in smaller denser LDL and smaller HDL, findings also observed in this thesis. It is believed that cholesterol ester transfer protein (CETP) mediates this remodelling when it becomes activated by increasing circulating triglycerides concentrations; CETP promotes transfer of triglycerides from triglyceride-rich particles (VLDL, chylomicrons and their remnants) to LDL and HDL receiving cholesterol in turn (Barter et al., 2003) . CETP was not however measured in this thesis but the changes in circulating lipid profiles in consistent with this mechanism.

Accumulation of VLDL and remnant lipoproteins, due to decreased lipolysis, is considered as one of the main causes of hypertriglyceridaemia in addition to increase production in ESRD (Tsimihodimos et al., 2011). It is well-known that dyslipidaemia is a risk factor for CVD in the general population (Lewington et al., 2007). In the ESRD population, controversy exists as to whether dyslipidaemia is a risk factor or not. It has been shown that dyslipidaemia is associated with CVD (Koch et al., 1997) while others failed to show this association (Shlipak et al., 2005).

Expression of lipid metabolism related proteins

The gene expression of lipid metabolism related proteins such as LPL, HSL, LDL-R, and C3 were investigated using human adipocytes culture. The current study indicates that their gene expression is altered when treated with uraemic lipoproteins (Table 7.1). This suggests a possible atherogenic role for uraemic lipoproteins. Lipoproteins structure, function, and metabolism seems to be altered

in renal disease (Mesquita et al., 2010). Therefore, dyslipidaemia in ESRD can be explained as a combination of both overproduction and low catabolism of lipoproteins, especially triglyceride-rich lipoproteins.

Adipokines and ESRD

There is currently limited information regarding the effect of uraemia on adipose tissue biology. Change in the adipose tissue composition, adipocytes number and the hormonal signalling either at the cellular level or at the organ level can all influence the secretory function of adipose tissue. The present results with 4 h treatment (acute effect) with uraemic serum from both types of dialysis, HD and PD, showed an inhibitory effect in adiponectin, leptin and IL-6 mRNA levels. This indicates that uraemic serum may alter the adipose tissue secretory function. Table 7.2 summarizes the results in chapter 5.

Changes in adiponectin

Although serum levels of adiponectin were high, its gene expression both *in vitro* in the present thesis, and *in vivo* by other groups, was found to be down-regulated in ESRD. A recent published study indicates that higher levels of adiponectin in ESRD may be explained in part by decreased clearance (Komura et al., 2010). The assumption is that the uraemic solutes in added sera altered *in vitro* adipocyte metabolism leading to a down-regulation of genes such as adiponectin. It is not clear which particular solute(s) may be responsible as such studies are beyond the scope of this thesis and should form the basis for further investigation. A feedback mechanism is a possible explanation whether adipokines themselves that are

increased due to lack of renal elimination in ESRD influence adipose tissue secretion.

The significance of the increased circulating total and high-molecular weight adiponectin in ESRD is not clear. Low plasma levels of adiponectin have been proposed as a new non-traditional risk factor for CVD in ESRD patients. However, whether those ESRD patients with high levels of adiponectin are protected from CVD is still not known. Many studies have supported low plasma levels of adiponectin as a risk factor for CVD in ESRD (Ignacy et al., 2005, Zoccali et al., 2002) while the opposite has been shown by other studies (Menon et al., 2006).

Changes in leptin

The present study showed that ESRD patients also have higher levels of circulating leptin with no change observed in the mRNA adipocyte levels. Leptin, in addition to its role in food intake and appetite regulation, has been proposed as proinflammatory adipokine (Iglesias and Diez, 2010) and found to be associated with inflammation (Nordfors et al., 1998). Although leptin is involved in appetite regulation, most studies failed to show any association between leptin and anorexia in ESRD (Heimburger and Stenvinkel, 2005). The high leptin observed in the present thesis is consistent with a pro-inflammatory consequence. Leptin is also considered to be a marker for fat mass. The relationship between the BMI and leptin in the present thesis is similar to the expected relationship in non-renal failure subjects and is consistent with a previous report in ESRD (Nishikawa et al., 1999). A recent study also demonstrated that higher plasma levels of leptin were associated with atherogenic lipid profile in ESRD patients (Kastarinen et al.,

2009b) .These data suggest that leptin could play a role in explaining at least in part the risk of CVD in ESRD.

Changes in IL-6 and MCP-1

In recent years it has become clear that cardiovascular disease due to atherosclerosis is a disease characterised by chronic low grade inflammation and there is a wealth of data supporting this key feature of atherosclerosis (Packard and Libby, 2008). Proinflammatory markers have also been found to be increased in ESRD. In the current study, described in greater detail in Chapter 5, IL-6 and MCP-1 gene expression were investigated *in vitro*. Interestingly, uraemic serum resulted in downregulation for IL-6 and no change in MCP-1 in normal adipocytes *in vitro*.

Although ESRD is associated with higher levels of IL-6, the current data showed no significant increase but a tendency towards high levels. The reason for the lack of increase in circulating IL-6 is debatable. On the other hand, it has been reported that normal or low levels of IL-6 could be present in ESRD patients, similar to what was seen in this thesis, and attributed to the genetic variation (Stenvinkel et al., 2002b). IL-6 plasma levels are considered as a mortality marker in ESRD, suggesting that the present cohort as a group were probably at lower risk (Kimmel et al., 1998). Patients in this study showed a significant higher level of adiponectin. IL-6 plasma levels were found to be inversely associated with adiponectin (Engeli et al., 2003) and this may be another explanation for low IL-6 mRNA levels. IL-6 is not produced exclusively by adipose tissue, about thirty percent of its plasma levels are produced by other cells and most of IL-6 that is produced by adipose tissue comes from stromal vascular fraction (Mohamed-Ali

et al., 1998). The studies in thesis only investigated adipocytes rather than the complete adipose organ.

Adipokine interaction in ESRD

Taken together these findings in this thesis supports the idea that adipose tissue *in vitro* respond differently when treated with uraemic serum. The higher levels of circulating adipokines can be both due a response to uraemia and the result of decrease renal clearance, the relative contributions being difficult to isolate. It is not known what the effect of the altered adipose tissue in terms of adipokines secretion means in terms of outcome in ESRD. The high leptin was not accompanied by high IL-6 suggesting that evidence for inflammation in terms of studied adipokines was less than expected; this was also balanced by markedly increased adiponectin expected to be anti-inflammatory. However, further work including investigation of an extended selection of adipokine expression is needed to fully characterise the 'uraemic' effect and should be considered for future studies.

Macrophage infiltration in adipose tissue

It is now widely accepted that adipocytes are not the main source of inflammatory markers secreted by adipose tissue. Other components including macrophages appear to be the cause of chronic inflammation in obesity (Weisberg et al., 2003). The present study, limited in numbers, demonstrates a tendency towards high macrophage infiltration in ESRD adipose tissue. Follow up work, enrolling more patients, would need to investigate the expression of macrophage markers at the gene and protein levels.

Future work

Time and resource constraints prevented a fuller investigation of ESRD and adipose tissue in this thesis. If these were not issues I would have considered these additional studies to inform on the relationship between adipose tissue and renal failure.

1. As clear differences in gene expression of adipokine have been shown in this thesis, however, further work including investigation of an extended selection of adipokine expression is needed to fully characterise the 'uraemic' effect and should be considered for future studies.

2. More detailed study design detailing the changes over time rather than 4 and 24 hours changes assessed in this thesis needs to be performed.

3. Protein expression both by western blots and free protein in the supernatant where appropriate should be carried out in future studies.

4. Further studies in ESRD, both by broadening the number of adipocyte genes (and proteins) and undertaking a mechanistic approach to clarifying the reasons for the changes observed would be necessary and informative.

5. The assumption is that the uraemic solutes in added sera altered *in vitro* adipocyte metabolism leading to a down-regulation of genes such as adiponectin. It is not clear which particular solute(s) may be responsible as such studies are beyond the scope of this thesis and should form the basis for further investigation.

6. The patients used for investigating adipocyte effects *in vitro* in this thesis were on multiple drug therapy. In order to exclude drug effects, an animal model of chronic renal failure could be developed and studied. Alternatively newly

diagnosed acute or chronic renal failure samples obtained before treatment is begun could be collected but will be challenging and there may be problems with obtaining sufficient numbers.

7. Samples for ESRD patients on haemodiafiltration would provide additional information about the effects of this form of renal replacement therapy on adipose tissue.

8. Samples from a range of renal impairment (CKD stages 1 to 5) could also be studied to further clarify ESRD effects.

9. Although this *in vitro* study demonstrated that uraemia altered adipose tissue but more extensive *in vivo* research is needed to confirm and extend these results.

Conclusion

In conclusion, an *in vitro* system to study the effect of uraemic lipoproteins and serum on adipose tissue nutrient and endocrine function through adipocyte gene expression was conducted and compared where available with the corresponding circulating protein measurements. More limited investigation of adipose tissue biopsies was also carried out. The results from the modulation of this *in vitro* adipocyte assay system not only serves as a convenient and unique model to study mechanisms of disease such as ESRD and its treatments but has so far also provided an initial insight that strongly suggests several mechanisms that could contribute to the CVD risk associated with ESRD. Further studies in ESRD, both by broadening the number of adipocyte genes (and proteins) and undertaking a mechanistic approach to clarifying the reasons for the changes observed would be necessary and informative.

Fraction		VLDL						LDL						HDL					
		H vs Pre-HD	H vs Post-HD	H vs PD	Pre-HD vs PD	Pre-HD vs Post-HD	*p*	H vs Pre-HD	H vs Post-HD	H vs PD	Pre-HD vs PD	Pre-HD vs Post-HD	*p*	H vs Pre-HD	H vs Post-HD	H vs PD	Pre-HD vs PD	Pre-HD vs Post-HD	*p*
LPL mRNA	4 h						NS		▼ $p <$ 0.05				0.02			▼ $p <$ 0.001			0.0034
	24 h						0.04				▼ $p <$ 0.001	▲ $p <$ 0.05	0.004						NS
HSL mRNA	4 h						NS				▼ $p <$ 0.05	0.032				▼ $p <$ 0.001			0.008
	24 h						NS						NS						NS
LDL-R mRNA	4 h						NS		▼ $p <$ 0.05	▼ $p <$ 0.05			0.0112						NS
	24 h						0.03						NS						NS
C3 mRNA	4 h						NS	▼ $p <$ 0.05	▼ $p <$ 0.001				0.0041			▼ $p <$ 0.05			0.012
	24 h						NS				▲ $p <$ 0.001		0.0115						NS

Table 7.1 Summary of the effect of lipoprotein fractions on mRNA levels of some key proteins in lipid metabolism

	Serum					
	H vs Pre-HD	H vs Post-HD	H vs PD	Pre-HD vs PD	Pre-HD vs Post-HD	ANOVA
Total Adiponectin			▲ $p < 0.001$	▲ <0.05		$p < 0.001$
HMW Adiponectin			▲ $p < 0.0001$	▼ $p < 0.001$		$p < 0.0001$
IL-6						NS
Leptin						$p < 0.05$

		Low dose						High dose					
		H vs Pre-HD	H vs Post-HD	H vs PD	Pre-HD vs PD	Pre-HD vs Post-HD	ANOVA	H vs Pre-HD	H vs Post-HD	H vs PD	Pre-HD vs PD	Pre-HD vs Post-HD	ANOVA
Adiponectin	4 h		▼ $p < 0.0001$	▼ $p < 0.0001$			$p < 0.0001$						NS
	24 h						NS						NS
IL-6	4 h	▼ $p < 0.0001$	▼ $p < 0.001$	▼ $p < 0.0001$			$p < 0.0001$						NS
	24 h						NS				▼ $p < 0.0001$		$p < 0.05$
MCP-1	4 h						NS						NS
	24 h						NS						NS
Leptin	4 h			▼ $p < 0.05$			$p < 0.0001$						NS
	24 h						NS						NS

Table 7.2 Summary of serum effects on adipokine

Appendices

Appendix 1

Part of the work in this thesis has been presented in the following posters:

1. Sultan Alouffi, Lakshminarayan Ranganath, Paul Trayhurn, Matthew Howse, Gordon Lowe, Ian Davis, I. Stuart Wood (2011). Lipoprotein subfraction profile of patients with End Stage Renal Disease. 4th Meeting Uremic Toxins and Cardiovascular Disease, Groningen, the Netherlands.

2. Sultan Alouffi, Lakshminarayan Ranganath, Matthew Howse, and I. Stuart Wood .(2011) Reduced gene expression of adiponectin in human primary adipocytes culture treated with uraemic serum. 4th Meeting Uremic Toxins and Cardiovascular Disease, Groningen, the Netherlands.

3. Sultan Alouffi, Lakshminarayan Ranganath, Paul Trayhurn, Matthew Howse, Gordon Lowe, Ian Davis, I. Stuart Wood (2011). Lipoprotein subfraction profile of patients with End Stage Renal Disease. 5th Saudi International Conference, the University of Warwick, United Kingdom.

4. Sultan Alouffi, Lakshminarayan Ranganath, Matthew Howse, and I. Stuart Wood. (2011) Reduced gene expression of adiponectin in human primary adipocytes culture treated with uraemic serum. 5th Saudi International Conference, the University of Warwick, United Kingdom.

Abstract 1

Lipoprotein subfraction profile of patients with End Stage Renal Disease

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OBJECTIVE: Patients with end-stage renal disease (ESRD) have an extremely high morbidity and mortality from cardiovascular disease (CVD). Dyslipidaemia is one of the best-known risk factors of CVD in ESRD although no reliable pattern of dyslipidaemia can be attributed to this disease. We have investigated differences in cholesterol distribution in lipoprotein fractions in ESRD patients, and with two types of dialysis treatment to describe changes in terms of their potential contribution to CVD risk.

METHODS: Iodixanol density gradients were employed to separate lipoproteins into VLDL, LDL, and HDL; lipid distribution was measured. Patients (n = 18) with ESRD (undergoing haemodialysis (n=11) or peritoneal dialysis (n=7)), and 9 healthy controls were recruited. Biochemical parameters and lipid plasma profile (cholesterol, LDL cholesterol and triglycerides) were assessed in all subjects.

RESULTS: Patients undergoing haemodialysis had significantly higher serum levels of triglycerides, and low HDL-cholesterol compared with the control group. Cholesterol distribution of lipoprotein fractions showed that haemodialysis patients had significant increased VLDL-cholesterol (p=0.0075) and presence of small, dense LDL-cholesterol than control participants. Peritoneal dialysis patients had significant increased VLDL-cholesterol (p=0.042), presence of small dense LDL-cholesterol and a shift in cholesterol to IDL.

CONCLUSIONS: ESRD is associated with dyslipidaemia, which is dependent on the type of renal replacement therapy. Peritoneal dialysis was associated with a more atherogenic lipoprotein cholesterol distribution. This lipoprotein cholesterol distribution may contribute to increased CVD in ESRD patients on renal replacement therapy.

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Abstract 2

Reduced gene expression of adiponectin in human primary adipocytes culture treated with uraemic serum

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OBJECTIVE: End stage renal disease (ESRD) is accompanied by increased cardiovascular disease (CVD) morbidity and mortality. Despite renal replacement therapy being employed ESRD is associated with increased complications including inflammation and dyslipidaemia. Adiponectin, a major adipokine, is known to demonstrate anti-inflammatory properties. We have investigated whether uraemic serum influences adiponectin expression in a human primary adipocyte culture system.

METHODS: A cohort of 18 patients with ESRD (haemodialysis and peritoneal dialysis) and 9 healthy (control) were analysed in a prospective cross-sectional study. Single blood samples were taken pre- and post-dialysis. Human adipocytes were incubated for 4 and 24 hours in culture medium containing serum from either healthy (control) or patients with ESRD (low and high dose). Serum concentrations of total adiponectin and high molecular weight adiponectin were measured by ELISA. Other biochemical parameters (cholesterol, LDL cholesterol, and triglycerides) were also assessed at the same time. Gene expression of adiponectin was quantified using the real-time polymerase chain reaction.

RESULTS: Serum concentrations of total adiponectin and high molecular weight adiponectin were significantly higher in the ESRD versus control group ($p=0.017$). Gene expression of adiponectin was lower in ESRD patients compared with healthy control at low dose but not with high dose serum treatment.

CONCLUSIONS: These results provide an initial insight in understanding the putative role of adipose tissue contributing to the associated of CVD risk in CKD patients. Our data suggests that normal adipocyte function is altered when exposed to an uraemic environment *in vitro*.

Appendix 2 Clinical Characteristics of studied patients

Patient No	Gender	Age	ESRD causes	CVD	Co-morbidity	Dialysis duration	Drug
HD1	Male	42	IgA nephropathy		Hypertension	7 months	Allopurinol, Minoxidil, Atenolo, Sodium Bicarbonate, Aranesp
HD2	Male	53	IgA nephropathy		Hypertension	17 months	Aranesp, Renagel, Ferrous sulphate, Bumetanide,Prednisolone, Candesartan, Sodium Bicarbonate, Residronate, Ezetimibe, Bisoprolol, Alfacalcidol, Atorvastatin
HD3	Male	46	Glomerulonephritis	Atrial fibrillation, Strokes	Obesity, endocarditis, hypertension	4 years	Renagel, Calcidol, Sodium Bicarbonate, Cinacalcet, Ranitidine, Paracetamol, Bisoprolol, Aspirin, Alucaps, Vitamin C, Zinc, Loperamide, Warfarin
HD4	Male	55	Chronic glomerulonephritis		Failed renal transplant, hypertension	18 months	Alfacalcidol, Renagel, Sodium bicarbonate, Omeprazole, Prednisolone, Magnesium, glycerophosphate, Neoral ,Ketovite, Allopurinol,Frusemide
HD5	Male	49	Proliferative glomerulonephritis	Myocardialinfarction, atrial fibrillation	Failed renal transplant, splenectomy, hypertension	19 years	Alfacalcidol, Alucap, Dispersible, Aspirin, Atorvastatin, Lansoprazole, Penicillin, Sodium Bicarbonate, Warfarin, Sotalol, Renagel, Amlodipine
HD6	Male	57	Renovascular Disease	Atherosclerosis		1 months	LANTHANUM CARBO, Alfacalcidol, Sodium Bicarbon, Folic Acid,Tramadol , Tamsulosin ,Chlorpheniramin, Zopiclone,Atorvastatin, Thiamine, Frusemide ,Allopurinol,

							Lansoprazole, Rosuvastatin, Salbutamol, Aspirin
HD7	Male	55	Polycystic kidney		Failed renal transplant, hypertension	6.5 years	Aranesp, Alfacalcidol ,Venofer ,Amlodipine ,Cinacalcet ,Tramadol ,Sodium Bicarbon , Lansoprazole ,Renegel ,Calcichew ,Piriton ,Immodium ,Allopurinol ,Co-Proxamol ,CyA (Neoral)
HD8	Male	52	Small kidneys	Atrial fibrillation	Failed renal transplant, thyrotoxicosis , skin cancer, hypertension	9 years	Alucap, Folic Acid, Propylthiouracil, Omeprazole, Digoxin, Bisoprolol, Amlodipine, Renagel, Atorvastatin, Sodium Bicarbonate Alfacalcidol, Aranesp, Venofer
HD9	Male	49	Polycystic kidney		Obesity, hypertension	3.5 years	Alfacalcidol , Lansoprazole, Renegel, Calcium Acetate, Amitriplyline, Frusemide, Sodium Bicarbonate
HD10	Male	48	Focal segmental glomerulosclerosis		Obesity, hypertension	5 years	Esomeprazole, Sodium Bicarbonate, Aranesp, Alfacalcidol
HD11	Male	40	Small kidneys	Myocardial infraction	Hypertension	13 years	Sertraline, Hypromellose, Flixonase , Aranesp , Minoxidil , Lisinopril , Diazepam , Quinine Sulphat
PD1	Male	50	unknown		Hypertension		
PD2	Male	75	Chronic glomerulonephritis	Myocardial infraction	Hypothyroidism, hypertension	3 months	Thyroxine , Ferrous sulphat , Adizem , Alfacalcidol , Allopurinol , GTN

						, Simvastatin , Aspirin
PD3	Male	38	Diabetic nephropathy	Diabetes mellitus, hypertension	3 months	Insulin, Lispro, Angitil, Simvastatin, Omeprazole, Sodium Bicarbonate, Calcium, Frusemide, Doxazosin, Ferrous Sulphate , Aranesp
PD4	Male	59	Hypertensive nephrosclerosis	Bronchiectasis,hypertendion, hypertension	3 years	Aranesp ,Venofer ,Atorvastatin ,Allopurinol ,Sodium Bicarbonate , Alfacalcidol ,Calcium Acetate , Renegel , Senna , Lactulose
PD5	Male	52	Single small kidney	Failed renal transplant, hypertension. hypogammaglobulinaemia	3.5 years	Allopurinol, Amlodipine Calcitriol ,Calcium Acetate , Carbocisteine , Aranesp , Doxazosin , Folic Acid , Frusemide , Metoprolol , Omeprazole , Prednisolone , Senna , Sevelamer , Simvastatin , Sirolimus
PD6	Male	35	Focal segmental glomerulosclerosis	Hypertension	2 years	Sodium Bicarbonate , Rosuvastatin , Aranesp , Calcichew , Lisinopril
PD7	Male	40	Alport syndrome	Hypertension	2.5 years	Calcium, Resonium, Atenolol, Amlodipine, Alfacalcidol,Adcal, Sobium Bicarbonate, Lisinopril
T1	Male	54	Diabetic nephropathy	Obesity, failed renal transplant	18 years	Aranesp, Mont,Venofer, Alfusozin , Frusemide , CyA (Neoral), Alfacalcidol, Pantoprazole, Novorapid, Lantus,Cinacalcet, Aspirin, Tramadol, Atorvastatin, Allopurinol, Sodium Bicarbon,Ventolin

T2	Male	64	Hypertensive nephrosclerosis	Hypertension	2.5 years	Finasteride, Tacrolimus, Lisinopril, Tamsulosin, Nifedipine, Alendronic Acid, Prednisolone, Alfacalcidol, Azathioprine, Sodium Bicarbonate, Calcium Carbonate
T3	Male	52	Failed renal transplant	Umbilical hernia repair, Lymphoma	4 years	Allopurinol, Amlodipine Calcitriol ,Calcium Acetate ,Carbocisteine ,Aranesp ,Doxazosin ,Folic Acid ,Frusemide ,Metoprolol ,Omeprazole ,Prednisolone ,Senna ,Sevelamer ,Simvastatin ,Sirolimus
T4	Male	65	Obstructive uropathy, kidney cancer	Failed renal transplant	5 years	**Sodium Bicarbonate,folic acid, Aranesp, Simvastatin, Minoxidil, Bisoprolol, Cinacalcet, sevelamer**
T5	Male	25	Hypertensive nephrosclerosis, kidney cancer		3 years	Ferrous Sulphate, Renagel, Frusemide, Diltiazem, One Alpha Calcidol
T6	Male	41	Hypertensive nephrosclerosis		18 months	Calcium Acetate , Sodium Bicarbonate ,Lercanidipine ,Aranesp , Warfarin

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