Lipoprotein lipase activity is reduced in dialysis patients.  
Studies on possible causal factors.

Akademisk avhandling

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Lipoprotein lipase activity is reduced in dialysis patients. Studies on possible causal factors

ABSTRACT

Cardiovascular disease is a major cause of mortality and morbidity in patients on chronic haemodialysis (HD). One main contributing factor is renal dyslipidaemia, characterized by an impaired catabolism of triglyceride (TG)-rich lipoproteins with accumulation of atherogenic remnant particles. The enzyme lipoprotein lipase (LPL) is a key molecule in the lipolysis of TG-rich lipoproteins into free fatty acids. The activity of LPL is reduced in HD-patients. This study was performed to elucidate various conditions and factors that may have an impact on LPL-related lipid metabolism.

I. The functional pool of LPL is located at the vascular surface. The enzyme is released by heparin and low molecular weight heparins (LMWH) into the circulating blood and extracted and degraded by the liver. Heparin and LMWH are used for anticoagulation during HD to avoid clotting in the extracorporeal devices. This raises a concern that the LPL system may become exhausted by repeated administration of LMWH in patients on HD. In a randomized cross over designed study twenty patients on chronic HD were switched from a primed infusion of heparin to a single bolus of LMWH (tinzaparin). The LPL activity in blood was higher on HD with LMWH at 40 minutes but lower at 180 minutes compared to HD with heparin. These values did not change during the 6-month study period. With heparin a significant TG reduction was found at 40 minutes and a significantly higher TG value at 180 and 210 minutes than at start. TG was higher during the HD-session with tinzaparin than with heparin. Our data demonstrate that repeated HD with heparin or with LMWH does not exhaust the LPL system in the long term but does disturb the LPL system and TG metabolism during every HD session.

II. In this study HD patients were compared with patients on peritoneal dialysis (PD) in a case control fashion. PD patients showed the same reaction of the LPL system to LMWH as HD patients. This confirmed that both HD and PD patients had the same, reduced, heparin-releasable LPL pool. The main difference was that in PD patients the TG continued to be cleared effectively even at 180 minutes after the bolus of LMWH injection. This may be due to a slower removal of the released LPL by the liver in PD patients.

III. In recent years, citrate (Citrasate) in the dialysate has been used in Sweden as a local anticoagulant for chronic HD. We performed a randomized cross over study that included 23 patients (16 men and 7 women) to investigate if citrate in the dialysate is safe and efficient enough as anticoagulant. The study showed that citrate anticoagulation eliminated the need of heparin or LMWH as anticoagulation for HD in half of the patients. However, individual optimization of doses of anticoagulants used together with citrate have to be made.

IV. Recently angiopoietin-like proteins, ANGPTL3 and 4 have emerged as important modulators of lipid metabolism as potent inhibitors of LPL. Twenty-three patients on chronic HD and 23 healthy persons were included as case and controls to investigate the levels of these proteins in plasma of HD-patients and to evaluate if HD may alter these levels. The data showed that plasma levels of ANGPTL3 and 4 were increased in patients with kidney disease compared to controls. This may lead to inactivation of LPL. High flux-HD, but not low flux-HD, reduced the levels of ANGPTL4, while the levels of ANGPTL3 were not significantly influenced. On HD with local citrate as anticoagulant, no LPL activity was released into plasma during dialysis in contrast to the massive release of LPL with heparin (LMWH). Citrate HD was not associated with a significant drop in plasma TG at 40 minutes, while both HD with citrate and heparin resulted in significantly increased TG levels at 180 minutes compared to the start values.

Conclusions: Citrate as a local anticoagulant during haemodialysis eliminates the need of heparin or LMWH in about half of the HD patients. Citrate does not induce release of LPL from its endothelial binding sites. We have shown that although HD with heparin causes release of the endothelial pool of LPL during each dialysis session, the basal pool is similarly low in PD patients that do not receive heparin. This indicates that the LPL pool is lowered as a consequence of the uraemia, per se. One explanation could be the increased levels of ANGPTL3 and 4. HD with high flux filters can temporarily lower the levels of ANGPTL4. Further studies are, however, needed to understand why LPL activity is low in patients with kidney disease.
KEY WORDS: Haemodialysis, lipoprotein lipase, ANGPTL3, ANGPTL4, citrate dialysate, dialyzer.

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

ORIGINAL PAPERS
I. Dana Mahmood, Maria Grubbström, Lennart DI Lundberg, Gunilla Olivecrona, Thomas Olivecrona, Bernd G Stegmayr
   Lipoprotein lipase responds similarly to tinzaparin as to conventional heparin during hemodialysis.
   BMC Nephrology 6; 11-33, 2010.

II. Dana Mahmood, Solveig Nilsson, Gunilla Olivecrona, Bernd Stegmayr
    Post-heparin lipoprotein lipase activity is similar in patients on
eritoneal dialysis compared to patients on haemodialysis.
    Submitted manuscript.

III. Bernd Stegmayr, Per Jonsson and Dana Mahmood
    A significant proportion of patients treated with citrate containing dialysate need
    additional anticoagulation
    International Journal of Artificial Organs. In publication.

IV. Dana Mahmood, Elena Makoveichuk, Solveig Nilsson, Gunilla Olivecrona, Bernd Stegmayr
    Response of angiopoietin-like proteins 3 and 4 to haemodialysis.
    Manuscript.

Paper I and III are printed with permission from the BMC Nephrology and International Journal of Artificial Organs.
ABBREVIATIONS

ANGPTL  angiopoietin-like protein
Apo      apolipoprotein
CDC      central dialysis catheter
Da       Dalton
FFA      free fatty acids
HD       haemodialysis
HDF      haemodiafiltration
HDL      high density lipoprotein
iCa      ionized calcium
IDL      intermediate density lipoprotein
LDL      low density lipoprotein
LMWH     low molecular weight heparin
LPL      lipoprotein lipase
PD       peritoneal dialysis
PTH      parathyroid hormone
RRT      renal replacement therapy
TG       triglyceride
UF       ultrafiltrate
UF-heparin unfractionated heparin
VLDL     very low density lipoprotein
INTRODUCTION

Background
The kidneys are organs that have important regulatory roles. They work as a natural filter of the blood and remove waste products and excess water. They have homeostatic functions such as regulation of electrolytes, maintenance of acid-base balance, and regulation of blood pressure. The kidneys also produce hormones including erythropoietin and renin. Renal failure is a condition in which the kidneys fail to purify blood from toxins and waste products. In severe renal failure renal replacement therapy (haemodialysis, peritoneal dialysis, and kidney transplantation) is essential for survival. According to the Swedish renal registry (1) there are about 8500 patients that have renal replacement therapy (RRT) in Sweden. Two-thirds of these are men. For the whole country the prevalence in RRT is 904 individ per million population (Dec. 31, 2010), while the incidence of RRT over the last 15 years was about 125 patients per million inhabitants per year. Fifty-six percent of all patients with RRT have a kidney transplant. For dialysis patients, 78% have haemodialysis and 22% have peritoneal dialysis (PD). The annual increase of patients with RRT is approximately 2-3%. Glomerulonephritis and diabetes mellitus are the most frequent causes of uraemia among the patients receiving RRT. The annual mortality for patients on dialysis has been about 22% over the last five years (From the Swedish renal registry 2010-12-31).

The main cause of morbidity and mortality in dialysis patients is cardiovascular disease (1). Cardiovascular diseases account for about 50% of deaths and 30% of hospitalizations in most registries (2). The reported cardiovascular death rates in patients receiving dialysis are
substantially higher than in the general population (almost 40 times greater). Cardiovascular mortality in end stage renal disease is particularly high after acute myocardial infarction, but it is also elevated in end stage renal disease patients with other forms of atherosclerotic vascular disease (e.g., chronic coronary artery disease, stroke, transient ischaemic attacks, and peripheral arterial disease) (3). There are several factors that are believed to contribute to increased incidence of cardiovascular mortality. One such factor is dyslipidaemia in dialysis patients which is thought to intensify atherosclerosis and vascular diseases. Accumulating data indicate that dialysis patients have atherogenic lipid abnormalities. Although low density lipoprotein cholesterol (LDL-C) levels in patients who undergo haemodialysis are normal or near normal, there are increased levels of oxidized LDL-C, triglycerides, and lipoprotein(a) [Lp(a)] as well as decreased levels of high density lipoprotein cholesterol (HDL-C). The triglyceridaemia in haemodialysis patients can be explained by the presence of triglyceride-rich very low density lipoprotein (VLDL) rather than an overproduction of VLDL (4, 5). Another important factor that contributes to increased morbidity and mortality in dialysis patients is malnutrition. Signs of protein-energy malnutrition are common in maintenance haemodialysis patients and are associated with increased morbidity and mortality (6). Cross-sectional analyses of patients in dialysis units indicate that the signs and symptoms commonly attributed to malnutrition occur in 50% or more of these patients (7). Causes of malnutrition in haemodialysis patients are numerous and various. One such possible cause is that patients on haemodialysis cannot utilize their lipids properly due to lipoprotein lipase (LPL) dysfunction (8, 9).
LPL activity in HD

**Lipid metabolism**

Fat (triglycerides) is a significant source of energy. On the average forty percent of calories derive from triglycerides (TG). Triglycerides are transported in blood in the core of lipoproteins (chylomicrons and VLDL). Ninety-five percent of their energy content is contributed by their fatty acids (5% by glycerol). Lipoprotein lipase (LPL) and hepatic lipase are key enzymes in the breakdown of triglycerides (10).

Dietary fat is carried in chylomicrons, and endogenous (hepatic) fat in VLDL to peripheral tissues where LPL hydrolyses the TG for energy supply or storage (Figure1).

Chylomicron remnants are removed from plasma by the liver (11, 12). All chylomicrons usually disappear from the circulation within 12–14 h after a fatty meal (12). VLDL remnants (called intermediate density lipoproteins, IDL) are either removed by the liver or converted to LDL(11), the main cholesterol- carrying particles. LDL-cholesterol either provides cholesterol to peripheral tissues or is removed by the liver. LDL is a major source of cholesterol, which is important for the synthesis of steroids and membranes or bile acid (13). HDL acts as a reverse cholesterol transport pathway returning cholesterol from cholesterol-replete tissues to the liver for interchange with other lipoproteins (10).
Lipoprotein lipase (LPL)

LPL is a member of the lipase family that includes hepatic lipase, pancreatic lipase and LPL itself (14). The lipases are encoded by genes that share structural similarities and are therefore likely to be derived from a common ancestral gene(14). LPL is produced mainly in the adipose tissue, cardiac and skeletal muscles, and the lactating mammary gland (15). LPL, from sites of synthesis,
LPL activity in HD

will be translocated to functional binding sites on the surface of vascular endothelial cells where it is anchored to heparan sulphate proteoglycans at the luminal surface. Lipoprotein lipase (LPL) is a lipolytic enzyme that mainly hydrolyses the TG in circulating chylomicrons and very low density lipoproteins thereby providing non-esterified fatty acids and 2-monoacylglycerol for tissue utilisation. It was first discovered in 1943 by Paul Hahn and was named clearing factor (16). In 1966 apolipoprotein C-II was found to be an essential cofactor for the action of LPL (17), which is mainly hydrolysis of the TG in circulating chylomicrons and very low density lipoproteins thereby providing non-esterified fatty acids and 2-monoacylglycerol for tissue utilisation. On the other hand fatty acids and apoC-III acts as LPL inhibitors. Inhibition of LPL by fatty acids is considered to be a feedback control mechanism. Abnormalities in LPL function have been found to be associated with a number of pathophysiological conditions, including chylomicronaemia, dyslipidaemia, atherosclerosis, obesity, and Alzheimer’s disease (18-20). LPL is consisting of a larger amino-terminal domain (residues 1–312) and a smaller carboxy-terminal end (residues 313–448). The carboxy-terminal domain is required for binding to the lipoprotein substrate whereas the amino-terminal domain is responsible for catalysis (21-23). LPL has high affinity for heparin, and heparan sulphate proteoglycans (24). The human LPL gene is located on the short (p) arm of chromosome 8 at position 22 (25). The molecular weight of the catalytically active, non-covalent LPL-homodimer is about 100 kDa (26).

**Disturbances in the lipid profile (dyslipidaemia)**

In chronic renal failure there are major disturbances in the lipid profile. The main issue is
dysregulation of HDL and TG-rich lipoprotein metabolism. It is characterized by impaired maturation of HDL, impaired clearance of TG-rich lipoproteins and their atherogenic remnants, and thereby elevation of their plasma concentrations. These alterations of lipid profile in chronic renal failure are primarily thought to be due to down-regulation of lipoprotein lipase (LPL), hepatic lipase, and the VLDL receptor, as well as, up-regulation of hepatic acyl-CoA cholesterol acyltransferase (ACAT)(9). Furthermore, impaired HDL metabolism leads to disturbances of TG-rich lipoprotein metabolism. These abnormalities are aggravated by down-regulation of apolipoproteins apoA-I, apoA-II, and apoC-II in patients with chronic renal failure(9). Renal dyslipidemia is already present in early renal insufficiency and primarily seen in a characteristic apolipoprotein pattern but are not necessarily manifested as hyperlipidaemia (27). Dyslipidaemia in chronic kidney disease (CKD) is characterized by increased concentrations of intact and partially metabolized ApoB and ApoC-III-containing TG-rich lipoproteins in VLDL, IDL and LDL (9, 28). The characteristic apolipoprotein profiles of renal dyslipidaemia are increased levels of ApoC-III, a reduced ratio between ApoC-III in HDL and ApoC-III in VLDL and LDL and a reduced ApoA-I/ApoC-III ratio(28).

Together, these abnormalities may contribute to the risk of arteriosclerotic cardiovascular disease and may adversely affect progression of renal disease and energy metabolism in chronic renal failure (29, 30).

**Uraemic toxins**

Uraemic retention solutes accumulate in patients with end stage renal disease. These retained solutes are called uraemic toxins when they contribute to the uraemic syndrome(31). There are three major groups of uraemic toxins based on their chemical and physical characteristics(31):
• Small, water-soluble, non-protein-bound compounds, such as urea (at least 45).

• Small, lipid-soluble and/or protein-bound compounds (at least 25).

• Larger so-called middle-molecules (MW above 500 Dalton), such as beta2-microglobuli (at least 22).

Of these solutes, 68 are characterized by a molecular weight below 500 Dalton (Da). Among the 22 middle molecules, 12 (54.5%) have a molecular weight that exceeds 12,000 Da (31). Apart from the organic substances only a few substances like urea, oxalic acid, parathyroid hormone (PTH) and β2-microglobulin have an established role as uraemic toxins.

Recently angiopoietin-like protein 4 (ANGPTL4) has been emerging as a substance that accumulates in haemodialysis patients (32). Animal studies have shown that ANGPTL3 and 4 are potent inhibitors of the lipoprotein lipase (33, 34). ANGPTL 3 and 4 belong to a family of proteins that share a common modular structure consisting of an amino-terminal coiled-coil domain and a large carboxy-terminal fibrinogen/angiopoietin-like domain (35, 36).

**Haemodialysis (HD)**

Haemodialysis is one of the methods that eliminate waste products and remove excess fluid from the body (also called ultrafiltration) in patients with end stage renal disease. The principle of haemodialysis includes diffusion, convection and hydrostatic pressure across a semipermeable membrane called dialysis filter or dialyzer. The dialysis filter separates the blood from a dialysate solution on the other side. The dialysis solution is a solution of mineral ions, electrolytes and glucose. Small water-soluble waste products such as urea and creatinine, potassium, and phosphate diffuse through tiny holes in the membrane from the blood into the dialysis solution (dialysate). The concentration of sodium and chloride in the dialysate are as in normal plasma to
prevent any loss during HD, but the concentration of sodium bicarbonate is higher than that in plasma to correct for acidosis. The target is to remove low-molecular-weight toxins from the blood and at the same time normalize the plasma concentration of molecules that may be altered in renal failure (37). Haemodialysis utilizes also counter-current flow. That means the dialysate is flowing in the opposite direction to blood flow in the dialyzer. Through this mechanism the system maintains the concentration gradient across the membrane at a maximum and the dialysis efficiency is increased. During dialysis free water can be removed (ultrafiltration) by altering the hydrostatic pressure of the dialysate compartment, causing free water and some dissolved solutes to move across the filter. This is necessary since most patients have none or strongly reduced urinary output. The reduced urinary output results in weight gain of fluid between the haemodialysis sessions. If this water is not removed the patient will suffer from progressive oedema including pulmonary oedema and high blood pressure (38).

Almost all dialyzers in use today are of the hollow-fiber mode. They have different pore sizes. Dialysis filters with smaller pore size are called low-flux dialyzer and those with larger pore size are called high flux dialyzer. The high flux membrane will enhance the convective removal of uraemic toxins through increased solvent drag (37, 39). The cut-off for a low flux membrane is about 5000 Da and for a high flux membrane it is about 15000 Da, according to the manufacturers (Gambro, Lund, Sweden and Fresenius Medical Care, Bad Homburg, Germany). The membranes with higher cut-off allow larger molecules to be removed.

Today’s dialyzer is commonly made from synthetic materials, polymer-based, which are more biocompatible and activate complement system to a lesser degree than the cellulose dialyzers used previously (40).

During chronic haemodialysis the access to blood is usually achieved from an arterio-venous
fistula, first developed by Brescia et al. in 1966 (41), or from a central venous dialysis catheter. The patient’s blood is pumped from the access through plastic tubes at a rate of 200-400 ml/min, into the dialysis filter and then the processed blood flows back into the patient’s blood circulation. The treatment procedure lasts about 4-5 hours, to be repeated three to four times per week. To prevent clotting within the dialyzer, unfractionated heparin (UF-heparin) in the past, and nowadays more often a bolus of low-molecular weight heparin (LMWH) is used for this purpose. In the counter side of the dialyzer the dialysis fluid passes the dialyzer in the opposite direction to the blood at a rate of about 500 ml/min.

The choice of dialysate composition is important in haemodialysis prescription in order to restore an adequate body electrolytic concentration and acid-base equilibrium. The main function of the dialysate is to remove waste material from the blood and to keep useful material from leaving the blood. Electrolytes and water are some materials included in the dialysate so that their levels are adjusted to that in the blood and body. The use of acetate-based dialysate in the past caused more vascular instability (42), formation of ketone-bodies (43) and less compensatory peripheral vasoconstriction (44). In Sweden, the most used dialysate nowadays is bicarbonate-based, which is well tolerated by HD patients (Table 3). Dialysis concentrates need acidification to prevent the formation of calcium and magnesium precipitates in dialysate. Acidification of bicarbonate-based dialysate is traditionally done with acetic acid which forms acetate in the final dialysate. The traditional systemic anticoagulation by UF-heparin or LMWH during haemodialysis may, beside increase the risk for bleeding, disturb the normal lipid metabolism because heparin releases LPL from its normal binding sites at the vascular endothelium (8, 45).
Peritoneal dialysis (PD)

In addition to haemodialysis there is another type of dialysis, which could be an alternative to haemodialysis, called peritoneal dialysis. In this case the peritoneal membrane is used as a native semi-permeable membrane that allows water and dissolved substances (electrolytes, urea, glucose, other small molecules and albumin) to be exchanged from the blood through diffusion, convection and osmosis. In this process the dialysis fluid (dialysate) is introduced through a permanent tube (peritoneal catheter) into the abdomen and exchanged regularly either throughout the night while the patient is asleep (i.e., automated peritoneal dialysis, APD; it involves the use of a peritoneal dialysis machine) or via regular exchanges throughout the day (continuous ambulatory peritoneal dialysis, CAPD). The peritoneal catheter (dialysis access) is surgically inserted with one end in the abdomen and the other protruding from the skin (46). The dialysate fluid (peritoneal dialysate) is introduced into the abdomen during 10 to 15 minutes. The total volume is usually between 2 – 2.5 litres. The fluid remains in the abdomen and waste products diffuse across the peritoneum from the underlying blood vessels. After a variable period of time, depending on the treatment, the dialysate is removed and replaced with a fresh dialysate. The time the solution remains in the abdomen between exchanges is called the dwell time. In automated peritoneal dialysis (APD) this exchange occurs, approximately hourly, automatically during the night while the patient is sleeping, whereas in continuous ambulatory peritoneal dialysis (CAPD) the patients themselves manually exchange the dialysate 4-5 times per day (47, 48). Unlike haemodialysis, patients on peritoneal dialysis do not need to travel to a dialysis centre for their treatment. Instead, after being trained at a dialysis centre, they will do their treatment at home. These patients do not need anticoagulation to perform their treatment.
AIMS OF THE PRESENT STUDY

The general aim of this study was to investigate to what extent LPL activity and TG metabolism is reduced in dialysis patients, and to study possible causal factors. In addition, the study included an approach to find a better way of dialysis to limit the negative effects upon the LPL-system that appears to be induced by uraemia and standard HD. Factors that disturb the LPL-system contribute to dyslipidaemia and increase the risk for cardiovascular disease.

The specific aim of each study:

**Study I**  The aim of this study was to explore if the LPL system gets exhausted by repeated administration of low molecular weight heparin in chronic HD-patients. In addition, the aim was to find out whether heparin or tinzaparin had the least negative effects on the LPL-system and on plasma triglyceride metabolism.

**Study II**  The aim of this study was to see if the amount of endothelial (heparin-releasable) LPL differed between patients on PD compared to those on HD, and to further clarify if the HD process has a more negative impact on the LPL pool.

**Study III**  The aim of this study was to investigate if citrate-containing dialysate was safe and efficient enough as the sole anticoagulation for patients on chronic HD.

**Study IV**  The aim of this study was to investigate the levels of the LPL controlling proteins ANGPTL3 and 4 in plasma of HD-patients and to evaluate if haemodialysis with various dialyzers or standard versus citrate anticoagulation may alter these levels.
SUBJECTS AND METHODS

Paper I

Twenty patients (12 men and 8 women) who had been on chronic HD for at least 3 months were studied. The demography of the study is given in Table 1. The mean age of the patients was 64 ± 12.5 years. The reason for dialysis was diabetes mellitus in 6 patients (30%), vasculitis in 4 (20%), interstitial nephritis in 3 (15%), polycystic kidney disease in 2 (10%), glomerulonephritis in 2 (10%) and one each with nephrosclerosis, postrenal obstructive problems, and myeloma associated amyloidosis. Prior to the study all patients were on dialysis with primed infusion of UF-heparin (Leo Pharma, Ballerup, Denmark) as anticoagulant. During the latter part of the follow up period data from four of the patients were not collected. Two of the patients died (one due to progressive myeloma and the other due to congestive heart failure), and two patients ended the study due to transplantation with cadaveric kidneys. The ethical committee at Umeå University approved the study and informed consents were obtained from the patients (Dnr 01-256). From a practical clinical point of view the study was performed as a quality assessment investigation to investigate if a change from UF-heparin to a bolus of tinzaparin would be beneficial for the patients.
Table 1: Baseline data.

The blood and serum values (± 1 standard deviation, SD) were obtained before dialysis at the last day of routine UF-heparin treatment and before start of the tinzaparin period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (SD)</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight, kg</td>
<td>77.6 (18.3)</td>
<td>73.3</td>
</tr>
<tr>
<td>Haemoglobin, g/l</td>
<td>121 (4.7)</td>
<td>120</td>
</tr>
<tr>
<td>Albumin, g/l</td>
<td>37 (4.2)</td>
<td>38</td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>21 (5.0)</td>
<td>22</td>
</tr>
<tr>
<td>Creatinine, µmol/l</td>
<td>683 (252)</td>
<td>625</td>
</tr>
<tr>
<td>total Cholesterol, mmol/l</td>
<td>4.0 (1.0)</td>
<td>4.1</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/l</td>
<td>1.9 (0.7)</td>
<td>1.9</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/l</td>
<td>1.2 (0.4)</td>
<td>1.1</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>2.0 (1.1)</td>
<td>1.6</td>
</tr>
<tr>
<td>Homocystein, mmol/l</td>
<td>16 (6)</td>
<td>16</td>
</tr>
<tr>
<td>Kt/V</td>
<td>1.46 (0.32)</td>
<td>1.47</td>
</tr>
<tr>
<td>Urea reduction at dialysis, %</td>
<td>72.8 (5.5)</td>
<td>72.5</td>
</tr>
<tr>
<td>Ultrafiltration volume, litres</td>
<td>1.50 (1.10)</td>
<td>1.55</td>
</tr>
<tr>
<td>Ultrafiltration, % of body weight</td>
<td>2.1 (1.9)</td>
<td>2.0</td>
</tr>
<tr>
<td>UF-heparin total dose, Units</td>
<td>7573 (1533)</td>
<td>7500</td>
</tr>
<tr>
<td>Tinzaparin total dose, Units</td>
<td>6075 (2100)</td>
<td>5500</td>
</tr>
</tbody>
</table>

The design of the study was to randomize the patients into two groups. The first group (six men and four women) continued with heparin that was the standard (bolus + maintenance) as anticoagulation for another 6 months before cross-over to tinzaparin. This was performed to enable comparison of possible changes over time with the tinzaparin-group. After switch to tinzaparin those patients were not crossed-over back to heparin. The second group (six men and
four women) were switched to tinzaparin (Leo Pharma, Ballerup, Denmark) at the start of study and continued on tinzaparin. There was a run-in period (2-4 weeks) in each group to adjust the dose of tinzaparin adequately in accordance to manufacturer’s advice to clotting appearance of the dialyzers, by visual grading.

Figure 2 displays the design of the study.
UF-heparin was given at the start of HD as a bolus of approximately 50 units/kg body weight followed by a continuous infusion of 800-1200 units/hour. The doses were adjusted with the aim to keep the activated partial thromboplastin time within 40-90 seconds until the last 45 min of the dialysis. For those with central dialysis catheter (CDC) the infusion was kept until the end of the haemodialysis.

Tinzaparin was initially given as a bolus of 4500 anti-Xa units. If clotting was noticed in the system or in the capillaries of the dialyzer (by vision), or if there was a bleeding tendency, the dose was changed in steps of 500 units according to the manufacturer’s recommendations. This was done during the run-in period.

Dialysis sessions were for a median of 270 min (240 min in 9 patients, 270 in 6 and 300 min in 5 patients) and did not change significantly over the study period. Samples were taken from the arterial side (before the dialyzer) of the dialysis system and measurements were performed during a total of 102 HD events. The dialyzers used were of high flux efficacy and supplied by Fresenius Medical Care (Hechingen, Germany; F8HPS and FX80, n= 1 versus 4 patients) and Gambro (Lund, Sweden; PF140H and PF210H, n= 9 versus 6 patients). Each patient used the same type of dialyzer for the whole series and there was no reuse of filters.

The dry weight (target weight), the weight that a patient with normal urine production would have despite fluid intake, was followed throughout the study. In patients without urine production there will be retention of water between dialyses and weight increases (inter dialysis weight gain). The weight gain is considered to be excess water and is removed during dialysis (38, 49)

The body weight, the serum albumin and urea concentrations were stable over the observation period indicating that the patients were not malnourished.
Blood samples were drawn at the start of the study when the patients were still on heparin as anticoagulant. For those who continued with heparin anticoagulation for another 6 months, samples were drawn at 3 and 6 months. The 6-month sample was considered the end-of-heparin-period sample. After switching to tinzaparin a run in period was allowed to adjust the dose according to the manufacturer. When the dose was considered adequately adjusted (after approx. 2-4 weeks) start values were drawn and samples were then taken after 3 and 6 months. The patients were asked to fast overnight before blood sampling. Those with diabetes mellitus were allowed to ingest only a small amount of carbohydrates prior to the dialysis to avoid hypoglycaemia. Some patients refused to fast. They were told to keep the same eating habit in this regard throughout the experiment periods to allow paired comparisons. Approximately 1 hour after the start of dialysis all patients were allowed a light meal. This meal contained mainly carbohydrates and protein (i.e. sandwich and ham) and tea or coffee. A regular lunch was served after the dialysis.

Blood samples were taken directly from the arterio-venous fistula or from the arterial line of the central dialysis catheter before dialysis. Variables analysed before the start of the dialysis session were creatinine, urea, albumin, total cholesterol, LDL-cholesterol, HDL-cholesterol and TG.

Blood samples at the end of dialyses were obtained according to the Swedish guidelines for blood sampling after dialyses. This means that after the end of the haemodialysis session the dialysate flow is stopped and the blood flow is reduced to 100 ml/min for 15 seconds before stop of blood pump and disconnection from the patient. Most of the blood that resided in the tubes was returned to the patient. The patient was disconnected from the tubing system and the blood samples were taken from the arterial needle at the site of the arterio-venous fistula or arterio-venous graft or
LPL activity in the blood samples was measured as previously described after hepatic lipase activity had been inhibited by antibodies (51, see page 92).

To estimate the amount of LPL activity residing at vascular endothelial surfaces at the end of the dialysis with tinzaparin as anticoagulant, a bolus of UF-heparin (50 units/kg body weight, intravenously) was given at 180 min. Eleven of the patients accepted to participate in this second step of investigation. Blood samples were taken at 180, 210 and 240 min (i.e. 0, 30 and 60 min
after the bolus of UF-heparin). Long-term changes in TG were evaluated by comparing values measured during one year prior to the study and values measured during 1.5-2 years after the study.

**Paper II**

The study was a case-control study in which 16 PD patients (12 men and 4 women) were compared to 16 HD patients (controls) (12 men and 4 women). Table 2 shows baseline data for both groups.

**Table 2: Baseline and outcome data.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Haemodialysis</th>
<th>Peritoneal dialysis</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Median</td>
<td>Mean</td>
</tr>
<tr>
<td>Age, year</td>
<td>16</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>16</td>
<td>74.5</td>
<td>78.2</td>
</tr>
<tr>
<td>TG start, mmol/L</td>
<td>16</td>
<td>1.71</td>
<td>2.19</td>
</tr>
<tr>
<td>TG 40 min</td>
<td>15</td>
<td>1.26</td>
<td>1.89</td>
</tr>
<tr>
<td>TG 180 min</td>
<td>15</td>
<td>1.96</td>
<td>2.28</td>
</tr>
<tr>
<td>Diff TG 0-40 min, mmol/L</td>
<td>15</td>
<td>-0.3</td>
<td>-0.35</td>
</tr>
<tr>
<td>Diff TG 0-180 min</td>
<td>15</td>
<td>0.26</td>
<td>0.04</td>
</tr>
<tr>
<td>LPL 40 min, U/L</td>
<td>16</td>
<td>49</td>
<td>56.4</td>
</tr>
<tr>
<td>LPL 180 min</td>
<td>16</td>
<td>3.0</td>
<td>3.4</td>
</tr>
</tbody>
</table>
The mean age of the PD patients was 59 years (SD±13) and the mean age of the controls (HD patients) was 66 years (SD±12). The reason for peritoneal dialysis was: nephrosclerosis (n=6), diabetic nephropathy (n=4), IgA nephritis (n=4), polycystic kidney disease (n=1), and graft versus host nephropathy (n=1); and for patients on haemodialysis: diabetic nephropathy (n=5), vasculitis (ANCA positive, n=3), polycystic kidney disease (n=2), glomerulonephritis (n=2), interstitial nephritis (n=1), nephrosclerosis (n=1), amyloidosis (n=1) and hydronephrosis (n=1). The ethical committee at Umeå University approved the study and informed consents were obtained from the patients.

PD patients received approximately the same tinzaparin dose (75 unit/kg) intravenously as the bolus dose given to the HD patients during their normal dialysis sessions. Most of the LPL is expected to be released from its binding sites at the endothelial surfaces by this tinzaparin dose(51). After injection of a bolus dose of tinzaparin, blood samples were drawn for analysis of LPL activity at 40 minutes for all patients and at 180 minutes for all HD patients, but only 6 patients with PD accepted to stay another 140 minutes and thereby to allow sampling for analyses of LPL activity at 180 minutes. The TG were analysed before administration of tinzaparin, at 40 minutes and at 180 minutes.

Neither the HD nor the PD patients accepted to be fasting. Therefore all were allowed a light breakfast with a cup of tea or coffee and only carbohydrates and proteins. Approximately 60 minutes after the first sample another light meal was served. A standard lunch was served after the dialysis session. The PD fluids used contained glucose. In five patients icodextrin was used as PD fluid during the study since these patients used automated PD at night and icodextrin (containing a polymer of glucose; Baxter International Inc., Deerfield, IL, USA) as the first bag
during the day. Blood was collected in heparinised tubes on ice. The plasma was immediately collected and frozen after centrifugation.

**Paper III**

In a prospective randomized cross over study twenty-three patients on chronic HD (16 men and 7 women) were included. Their mean age was 62 years (range 18-87). Diabetes mellitus was present in 48% of the patients. All performed high flux HD (8 with on-line HDF, using the FX80 dialyzers, Fresenius MediCare, Bad Homburg, Germany). The same conditions were present for each patient during the dialyses performed in the study. One patient (male, 55 years), on the waiting list, received a kidney transplant after having performed the first series of the study, i.e. standard HD. His data are only present for calculations during the standard HD procedure.

The anticoagulation used during HD was either start with a bolus of LMWH (tinzaparin) or to start without LMWH and only use citrate in the dialysate (Citrasate 461, MTN, Neubrandenburg GmbH, Neubrandenburg, Germany, provided in Sweden by Scandinavian Medical, Kista, Sweden). The dialysate was either the standard, used at our department (Smartbag, Fresenius Medical Care, Bad Homburg, Germany) or Citrasate. The final concentrations of substances in the dialysates used are presented in Table 3.
Table 3: Concentration of various compounds in Citrasate versus Standard dialysates.

<table>
<thead>
<tr>
<th>Product</th>
<th>Measure</th>
<th>Citrasate® Dialysate</th>
<th>Standard Dialysate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>mmol/l</td>
<td>140</td>
<td>138</td>
</tr>
<tr>
<td>Potassium</td>
<td>mmol/l</td>
<td>2.0</td>
<td>2.0 – 3.0</td>
</tr>
<tr>
<td>Calcium</td>
<td>mmol/l</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>Magnesium</td>
<td>mmol/l</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Chloride</td>
<td>mmol/l</td>
<td>105.5</td>
<td>108.5</td>
</tr>
<tr>
<td>Acetate</td>
<td>mmol/l</td>
<td>0.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>mmol/l</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>mmol/l</td>
<td>37</td>
<td>32</td>
</tr>
<tr>
<td>Glucose</td>
<td>mmol/l</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

During standard dialysis a tinzaparin dose of approximately 75 units/kg body weight was used as a bolus before the start of the dialysis and the dose was adjusted to achieve adequate dialysis with minimal visual clotting. When Citrasate dialysis was performed no bolus dose of tinzaparin or other adjuvant anticoagulation was given before the start of HD. The study included analyses of subjective patency, ionized calcium (iCa) and urea reduction rate (48) between start and at 210
minutes of HD. Approval of the Ethics Committee in Umea was obtained as well as approval by the Swedish Product Agency. Patients were informed and consented to participate in the study. To perform citrate dialysis the dialysis devices needed adjustments by a technician as described in paper III.

Before the study started the patients performed one dialysis with the Citrasate dialysate and a 50% reduced dose of their regular bolus dose of tinzaparin at start. This was done to check for important clotting tendencies or side effects. Through this test series patients at risk for clotting were identified. Such patients were followed up more closely during the study dialysis.

**Paper IV**

The primary study included 23 (14 men and 9 women) patients who had been on chronic HD and 23 healthy controls (blood donors, 14 men and 9 women) that were matched for age and gender. The mean age of the controls was 65 years (SD± 14.8) and for the patients the mean age was 65 years (SD± 3.9). Blood samples were obtained from the 23 HD patients before dialysis and compared to samples from the 23 healthy controls matched for age and gender.

In the second part of the study, blood samples were drawn before start, at 40 minutes and 180 minutes after start during a HD session in 17 patients. A bolus dose of tinzaparin was given as anticoagulation at start of HD and there was no additional tinzaparin given during the 180 minutes period. The extent of ultrafiltration was at a mean of 1889 ml (SD± 1063.6 ml) with a median of 1700 ml. In 11 patients high flux dialyzer were used: four of them used Xenium 190 H (Baxter International Inc., Deerfield, IL, USA), five others used FX 80 H (Fresenius, Bad Homburg, Germany), one patient used FX1000H (Fresenius) and another used PF 210 H
(Gambro, Lund, Sweden). Six patients used low flux dialyzers (FX 10 L by Fresenius). The high flux dialyzer has a cut-off of about 15-20 kDa and that for low flux dialyzer is about 5 kDa. ANGPTL3 and ANGPTL4 were analysed before start, at 40 minutes and at 180 minutes of HD.

Blood samples for high sensitive C-reactive protein (CRP) were taken before and after HD, and S-albumin and triglycerides (TG) were analysed before the HD session. Blood sampling before dialysis was performed directly from the arterio-venous fistula/graft or from the arterial line of the central dialysis catheter. During dialysis the samples were taken on the arterial side of the tubing set, before the dialyzer. Serum or plasma samples were stored at -70° C until analysis.

In the third part of the study we investigated a group of patients treated alternately with local citrate for anticoagulation compared to their standard anticoagulation with a bolus tinzaparin. The study included 22 patients with chronic HD (15 men and 7 women). The design resulted in that the patients were their own controls. Each patient was dialyzed with either a tinzaparin bolus or with local citrate in the dialysate as anticoagulant (Table 5). This was a part of paper III. Laboratory data were used for analyses until, but not including, the time on dialysis when tinzaparin had to be added to the blood to avoid further clotting of the tubing system. Blood samples were obtained before start, at 40 and 180 min of dialysis for analysis of TG and ANGPTL3 and 4, while samples were taken for analysis of LPL at 40 minutes and 180 minutes of dialysis.

Only 7 patients were selected for LPL and ANGPTLs analysis out of those 11 who did not need any additional tinzaparin during citrate-dialysis. A release of LPL from endothelial binding sites was not expected during the citrate dialysis.

The ethical committee at Umeå University as well as the Swedish Product Agency approved the study. Informed consents were obtained from the patients.
Laboratory analyses

Lipoprotein lipase analyses

Blood samples for lipase activity were collected in heparinized tubes. They were immediately chilled on ice and centrifuged within 15 minutes. The plasma was frozen and stored at –70°C until analysis for LPL. LPL-activity was measured with an emulsion containing 100 mg soybean triglycerides and 10 mg egg yolk phospholipids per ml containing tritiated oleic acid–labeled triolein (prepared by courtesy of Fresenius-Kabi, Uppsala, Sweden). Hepatic lipase activity was inhibited by pre incubation of the plasma samples with immunoglobulins from a rabbit antiserum to human hepatic lipase. All assays were performed in triplicate and the mean value was used for calculations. A standard sample of human post-heparin plasma was run on each assay day and the value was used to calibrate for between-assay variation (51).

ANGPTL3 and 4 analyses

Concentrations of ANGPTL3 and 4 in plasma or serum were measured by ELISA kits from R&D Systems (Abingdon, UK; cat. Nr DY3829 and cat. Nr DY3485, respectively) with some modifications including incubation with capture antibodies for 4 h at 37 °C, dilution of samples in 1% BSA-0.1% Tween 20 followed by overnight incubation at 4°C and use of OPD (1,2-phenylenediamine dihydrochloride) tablets, from Dako (Glostrup, Denmark (S2045), as substrate. The reaction was stopped with 0.5 M \( \text{H}_2\text{SO}_4 \) (100 \( \mu \)l/well), and the absorbance was measured on a SPECTRAmax 340 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) as the
difference between readings at 490 nm (for the product) and 570 nm (for correction of optical imperfections in the plate).

For analyses of ANGPTL4 in ultrafiltrates, 25 ml were lyophilized, dissolved in 0.8 ml of distilled water and dialyzed at 4 °C against 10 mM phosphate buffer with 0.15 M NaCl (3 changes of buffer, 1.5 h each) using dialysis tubing with 3.5 kDa cut-off (Spectra/Por®, Spectrum Laboratories, Inc, DG Breda, The Netherlands). After dialysis the volume was adjusted to 1 ml with the buffer and precipitates (formed during dialysis) were removed by centrifugation (15 000xg, 30 sec at 4 °C). The supernatants were used for analysis of the amounts of ANGPTLs by ELISA.

**STATISTICS**

The data are presented as median or means ± SD, unless stated otherwise. All statistical analyses were performed using SPSS version 19 for Macintosh (SPSS, Inc., Chicago, USA). Student independent or dependent T tests were used when appropriate. When the material was small and the distribution was not normal, non-paired (Mann-Whitney) and paired (Wilcoxon) non parametric analyses were used. Wilcoxon’s paired rank test was preferred instead of parametric tests to reduce the risk for interaction by more or less skewed distribution. For analyses of correlation the Spearman test was used. Fishers test and Chi-2 analyses were used. A two tailed p-value of <0.05 was considered as significant.
RESULTS

Lipoprotein lipase responds similarly to tinzaparin as to conventional heparin during haemodialysis (paper I)

After injection of UF-heparin or tinzaparin a significantly high LPL-activity during dialysis was observed at 40 minutes and thereafter the LPL-activity was significantly reduced at 180 and 210 minutes (Figure 3).

Figure 3. Median LPL activities in plasma during dialysis with UF-heparin or tinzaparin.
There were no significant differences in the levels of LPL activity over the 6-month study period. The correlation between the levels of LPL-activity at 40 minutes at the start of tinzaparin and after 6 months on tinzaparin was strong ($r=0.76, p<0.01$). There was also a significant correlation between the tinzaparin and UF-heparin regarding the high LPL-activities at 40 minutes ($r=0.55, p<0.02$). The LPL-activity was higher at 40 minutes and lower at 180 and 210 minutes for tinzaparin compared to UF-heparin. There was a significant reduction of plasma TG during HD at 40 minutes ($p<0.001$). The TG values increased again at the end of dialysis (180 and 210 min) to almost the same values as at the start of dialysis before administration of UF-heparin/tinzaparin (Figure 4). The TG values had not decreased as much at 40 min and were higher at 180 and 210 min after tinzaparin than after UF-heparin.
Figure 4. Median change in plasma TG concentrations during HD with UF-heparin (open circles, filled line) or tinzaparin (filled square, dotted line).

Post-heparin LPL activity is similar in patients on PD compared to patients on HD (paper II).

There were no significant statistical differences in plasma LPL-activity at 40 min after injection of tinzaparin between PD and HD patients, which indicates that the LPL-pool is similar in both PD and HD groups. The activity of LPL was diminished, in the same way as in HD group, to almost basal level at 180 min in PD patients (Figure 5). However, there was a higher value for
median LPL-activity at 180 min for PD patients compared to HD patients. This difference was significant (p<0.005).

![Figure 5](attachment:image.png)

**Figure 5:** Median levels of LPL activity in plasma in age- and gender- matched PD (open square, hatched line) and HD patients (filled square, filled line) at 40 min and 180 min after receiving tinzaparin. The LPL activity in plasma before injection of tinzaparin is too low to be measured by the method and was therefore set to 0 (0 min).

The analyses of TG showed that at 40 min the level was reduced significantly compared to start values in both the PD (p<0.001) and the HD (p=0.004) groups. However, in HD patients the TG level after 40 min started to increase significantly to reach the baseline levels at 180 min (p=0.027). Such increase was not the case in PD patients where the TG level remained unchanged below the baseline at 180 min (Figure 6).
**Figure 6:** Median plasma TG concentrations in PD (open square, hatched line) and HD patients (filled square, filled line) immediately before (0 min) and at 40 min and 180 min after injection of tinzaparin.

PD patients had significantly greater reduction in TG (extent of change) from start to 40 min than HD patients (p<0.001) and remained so even at 180 min (p=0.001) (Figure 7).
Figure 7: Median change in plasma TG concentrations in PD (open square, hatched line) and HD patients (filled square, filled line) from immediately before (set 0 min) and at 40 min and 180 min after injection of tinzaparin.

A significant proportion of patients treated with citrate containing dialysate need additional anticoagulation (paper III)

Tinzaparin was used in a bolus dose at a median of 5000 Units (range: 2500-11000) for standard HD. Total calcium was at a median 2.31 mmol/l (1.88-2.78) and ionized calcium (iCa) was 1.25 mmol/l (1.08-1.28). In Citrasate dialysis there were no administration of tinzaparin at the start of
HD. The start value of iCa was 1.20 mmol/l (1.01–1.40). During Citrasate HD (Cit-HD) the iCa was slightly but significantly more reduced with time.

Median iCa after 210 min of HD was 1.03 for Cit-HD and 1.16 for standard-HD (p=0.001, Figure 8).

**Figure 8:** Median value of ionized calcium (iCa) during HD with standard HD (filled circles, filled line) and Citrasate-HD (open squares, filled line). The lowest iCa in any patient during Citrasate-HD is shown with a hatched line and open triangles.

There was a progressive reduction in median change in iCa during the whole Cit-HD (Figure 9).
**Figure 9:** Median change in ionized calcium during Citrasate-HD.

The dialyzers patency was graded by the extent of clot-filled capillaries. There were significantly more dialyzers that had clots formed during the Citrasate-HD compared to standard-HD (Table 4).
Table 4: Distribution of clot grade in percentage and relative risk (RR), confidence interval (CI) and p-value (P) if the relative risk could not be calculated.

<table>
<thead>
<tr>
<th>Clot grade</th>
<th>Citrasate HD N (%)</th>
<th>Standard HD N (%)</th>
<th>RR (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3 (13.6)</td>
<td>21 (95.5)</td>
<td>17.5 (2.6-119)</td>
</tr>
<tr>
<td>Mild</td>
<td>8 (36.4)</td>
<td>1 (4.5)</td>
<td>7.8 (1.2-50)</td>
</tr>
<tr>
<td>Moderate</td>
<td>7 (31.8)</td>
<td>0</td>
<td>P=0.0002</td>
</tr>
<tr>
<td>Severe, interrupted HD</td>
<td>4 (18.2)</td>
<td>0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Addition of tinzaparin during HD</td>
<td>7 (31.8)</td>
<td>0</td>
<td>P=0.0089</td>
</tr>
</tbody>
</table>

There were only 14% of dialyzers during Citrasate-HD that seemed to be without clots (by visual grading). In seven patients additional tinzaparin was given during Citrasate-HD due to clots growing in the venous chamber. All of the Citrasate dialyses that received additional tinzaparin were able to complete the prescribed HD session. The median dose of tinzaparin added during these dialyses was 40% of their standard dose (range 10-74%). Eleven of 22 patients (50%) did not need additional tinzaparin and could perform HD without problems. Five of these (45%) had moderate (grade 2-red) clots in the dialyzer after HD.
Response of angiopoietin-like proteins 3 and 4 to haemodialysis (paper IV)

The levels of ANGPTL3 and 4 were significantly higher in HD patients compared to healthy controls (p=0.001). The values for ANGPTL3 were significantly higher than ANGPTL4 for both HD patients and controls. During haemodialysis no significant changes were observed in the level of serum ANGPTL3. On the other hand there was a significant increase of the level of ANGPTL4 at 40 min (p=0.001), and thereafter a significant reduction at 180 min compared to the values at 40 min (p<0.001) and at start (p=0.013) during haemodialysis (Figure 10).

Figure 10: Median value of angiopoietin-like protein 3 (ANGPTL3) and 4 (ANGPTL4) in 17 HD patients (low and high flux dialyzer together) before start, at 40 minutes and 180 minutes of HD. ANGPTL3 (ns). ANGPTL4 (0-40 min, p= 0.001), (40-180 min, p= 0.001), (0-180 min, p= 0.013).
The impact of the type of dialyzer used during haemodialysis on elimination of ANGPTL3 and 4 was studied. There was a significant reduction of ANGPTL4 at 180 min compared to start value for those dialysed with a high flux filter (p=0.003). This significant decrease of ANGPTL4 at 180 min was not observed with low flux filters. With regard to ANGPTL3, there were no significant changes in the level of ANGPTL3 at 180 min compared to the start value during haemodialysis neither with low flux nor with high flux filters (Figure 11).

**Figure 11:** Median value of angiopoietin-like protein 3 (ANGPTL3) and 4 (ANGPTL4) in 17 HD patients. Low flux (L) and high flux (H) dialyses are shown separately (6 patients using a low flux and 11 a high flux dialyzer) before start, at 40 minutes and 180 minutes of haemodialysis. ANGPTL3 low flux dialyzer (ns). ANGPTL3 high flux dialyzer (0-40 min, ns), (40-180 min, p=0.016), (0-180 min, ns). ANGPTL4 low flux dialyzer (0-40 min, p=0.028), (40-180 min, p=0.028), (0-180 min, p=ns). ANGPTL4 high flux dialyzer (0-40 min, p=0.01), (40-180 min, p=0.003), (0-180 min, p=0.003).
In order to confirm this elimination of ANGPTL4 by high flux dialyzers we analysed the samples from ultrafiltrates (UF) and could detect ANGPTL4 in UF samples. On the contrary no ANGPTL3 could be traced in UF samples.

To study further the role of tinzaparin, we compared the levels of plasma ANGPTL3 and 4 when using local citrate instead of systemic tinzaparin for anticoagulation. With citrate no measurable LPL activity appeared in plasma. The concentration of ANGPTL3 also did not change. In contrast, there was a significant reduction of ANGPTL4 at 40 minutes (p=0.018) and at 180 minutes (p=0.018) compared to the start value (Table 5).
Table 5: Data on patients that were dialyzed with local citrate (Citrasate) as anticoagulant compared to heparin (tinzaparin). Values are mean ± SD.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HD with tinzaparin</th>
<th>HD with Citrasate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Age, years</td>
<td>22</td>
<td>62.7 (15.1)</td>
</tr>
<tr>
<td>Males, N</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Females, N</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>TG at start, mmol/L</td>
<td>22</td>
<td>1.95 (1.1)</td>
</tr>
<tr>
<td>TG at 40 min</td>
<td>14</td>
<td>1.57 (1.11)</td>
</tr>
<tr>
<td>TG at 180 min</td>
<td>22</td>
<td>2.06 (1.49)</td>
</tr>
<tr>
<td>LPL activity at 40 min (mU/mL)</td>
<td>7</td>
<td>65.28 (27.9)</td>
</tr>
<tr>
<td>LPL activity at 180 min (mU/mL)</td>
<td>7</td>
<td>9.28 (10.98)</td>
</tr>
<tr>
<td>ANGPTL3 before HD, ng/mL</td>
<td>7</td>
<td>335 (141)</td>
</tr>
<tr>
<td>ANGPTL3 at 40 min HD, ng/mL</td>
<td>7</td>
<td>344 (153)</td>
</tr>
<tr>
<td>ANGPTL3 at 180 min HD, ng/mL</td>
<td>7</td>
<td>301 (137)</td>
</tr>
<tr>
<td>ANGPTL4 before HD, ng/mL</td>
<td>7</td>
<td>254 (95)</td>
</tr>
<tr>
<td>ANGPTL4 at 40 min, ng/mL</td>
<td>7</td>
<td>242 (107)</td>
</tr>
<tr>
<td>ANGPTL4 at 180 min, ng/mL</td>
<td>7</td>
<td>184 (47)</td>
</tr>
</tbody>
</table>

*Levels were below the detection limit of the method. * a - p-value before versus 40 min; b - p-value before versus 180 min; c = p-value tinzaparin versus citrate; NS = not significant
DISCUSSION

Paper I

There were no substantial differences between UF-heparin and tinzaparin regarding their anticoagulation’s effect on the function of dialyzers. Previous studies have shown that LMWH has a lesser impact on LPL system compared to UF-heparin. However, in animal studies LMWH has been as effective as UF-heparin in releasing LPL from its binding sites at the endothelial surfaces into the blood, but LMWH has a decreased ability to delay the removal of LPL by the liver(52). In the present study the activity of LPL in the plasma was somewhat higher at 40 min after tinzaparin than after UF-heparin. This may be explained by a larger number of heparin molecules that was administered with the LMWH preparation, which could efficiently release the LPL. Tinzaparin that comprises of the shorter heparin chains may have a reduced capacity to retain the LPL in the circulating blood. This could explain the lower LPL-activity at 180 min after tinzaparin bolus compared to UF-heparin infusion. In a previous study the activity of LPL after dalteparin was only about one-third of that with UF-heparin (51). This difference between dalteparin and tinzaparin regarding LPL-activity could be explained by a larger molecular weight of tinzaparin that makes it structurally closer related to UF-heparin (53). LPL is normally bound at the vascular endothelium (54). Only a fraction of the TG-rich lipoprotein particles in the circulating blood are in contact with LPL at the endothelium (55). It was previously concluded that the level of TG is initially decreased after injection of UF-heparin or LMWH due to accelerated lipolysis through release of high amounts of active LPL to the circulating blood (56-58). We found that this early drop of plasma TG was less marked with tinzaparin than with UF-heparin. Later the continued release of LPL from the endothelium to the blood circulation and accelerated transport to the liver for degradation (59) creates a state of temporary depletion of the
lipase (8, 52). This could be the main explanation for the increase of plasma TG seen towards the end of the HD session and some hours thereafter. This rise in TG was more marked with tinzaparin compared to UF-heparin. In this regard the ability of the HD patients to utilize energy from free fatty acids through degradation of TG appears to be diminished during the later part of dialysis. Näsström et al demonstrated that there was a delay of the recovery of the LPL pool for several hours after a HD session (56). However, the LPL-system and lipoprotein metabolism seems to be fully recovered between dialyses sessions since there was no difference in the values for releasable LPL activity after administration of tinzaparin or UF-heparin throughout the 6 months study period, and the plasma lipid levels did not change during this period in the present study. Hence, repeated HD with tinzaparin or UF-heparin does not exhaust the LPL-system and does not explain the relatively low levels of heparin-releasable LPL activity in patients on chronic HD.

**Paper II**

This study showed that LPL-release from the vascular endothelium into blood circulation by tinzaparin was approximately the same in both PD and HD patients when compared at 40 min after injection of tinzaparin. At 180 min after tinzaparin the LPL-activity was significantly higher in PD patients compared to HD patients. This could possibly be explained by a slower uptake and degradation of LPL (or tinzaparin) by the liver in PD patients. Presumably as a result of that, the TG level continued to stay low at 180 min in PD patients. The difference in this respect between PD and HD patient was significant although data were obtained from only 6 PD patients at 180
LPL activity in HD

...min. The similar levels of LPL-activity at 40 min in PD compared to HD patients supported the view that repeated treatment with tinzaparin during HD is not the cause of the low levels of endothelial (heparin-releasable, between dialysis sessions) LPL activity in patients with chronic kidney disease. This LPL-system rather seems to be inhibited by factors due to the uraemia.

In both PD and HD patients there is an increase in the lipid and apolipoprotein mass in intermediate-density lipoprotein (IDL), and an enrichment of ApoCIII in VLDL, IDL, and LDL (60). The main difference in lipoprotein composition between PD and HD is elevation of ApoB in LDL in PD patients.

There may also be other factors that affect the levels of LPL activity. These include, reduced expression levels of glycosylphosphatidylinositol-anchored HDL-binding protein 1 (GPIHBP1) in several tissues. This was found recently in rats with induced renal failure (61). Another cause could be the presence of the high levels of LPL inhibitors in the blood of these patients, such as ANGPTL3 and 4 (according to paper IV), ApoCIII (62), and Apo-I in the pre-beta fraction of HDL (63). The result of my study shows that with regard to the endothelial pool of LPL activity PD is equivalent to HD. The results confirm that the endothelial pool of LPL is not exhausted during long term HD. LPL is likely to be rapidly restored at the endothelium as soon as the heparin or tinzaparin has been cleared from plasma.

**Paper III**

This study showed that chronic HD with local citrate in the dialysate as anticoagulation was able to be completed in 50% of the patients, without additional anticoagulant, although moderate clotting was found in 45% of this type of HD. The dialyses were prematurely interrupted in 18%
of the patients. A similar extent of clotting with citrate dialysis (up to 22%) was also found by others (64, 65). Intravenous citrate (regional administration) is used mainly as anticoagulation in the intensive care unit to prevent clotting during continuous veno-venous haemodialysis. For this the doses are higher and the decreased breakdown of citrate may be a problem in patients with impaired liver function. By using a local citrate as an anticoagulant directly at the surface of the dialyzer such risks are reduced. Since there were no obvious clinical side effects that could be related to the citrate in this study, this type of treatment can be used without additional tinzaparin in a selected group of patients. By using a limited addition of anticoagulation it is possible to complete prescribed HD session in another 30% of patients.

According to our experience during Citrasate HD a dose reduction of anticoagulation to 50% can be used as a first step. If clotting occurs in the dialyzer during this first step of procedure, a further reduction of anticoagulation during the next HD session needs caution, and a total withdrawal may be impossible. Such patient may need a maintenance dose of additional intravenous anticoagulant for local citrate HD sessions.

It is known that to achieve optimal prevention of clotting the ionized calcium needs to be reduced to levels of less than 0.2 mmol/l (66). On the other hand, the risk for severe hypocalcaemia is not present using the citrate concentration prescribed for the dialysate in this study in contrast to the regional citrate infusion. The latter model needs an adequate calcium infusion system to compensate for the more extreme changes in ionized calcium (66).
This study shows that ANGPTL3 and 4 were accumulated in HD patients to levels that are two and three fold higher than those found in age-matched healthy persons. Baranowski et al. reported a similar increase of ANGPTL4 in patients on HD (32). On the other hand, in a previous study the level of ANGPTL3 in pre-HD and HD patients was reported to be significantly lower than the healthy controls (67). This is in contrast to our results on ANGPTL3 and should be explored by further studies. The accumulation of ANGPTL3 and 4 in dialysis patients could either be due to impaired renal filtration or too extensive production of these proteins or to both. In studies in animals the production of ANGPTL3 and 4 were shown to be stimulated by several factors including hypoxia (68), chronic inflammation (69), and tumour (70). Craddock et al. observed that hypoxia can occur during the first hours of HD (71). We did not measure oxygenation, but noted that our patients did not have increased levels of CRP, indicating that inflammation was in most cases not an issue.

The study demonstrated that haemodialysis has no significant impact on the level of ANGPTL3 neither with low flux filter nor with high flux filter. On the other hand, during dialysis the ANGPTL4 was significantly increased at 40 min with both low and high flux dialyzers, and thereafter the levels were reduced significantly at 180 min with high flux dialyzer but not low flux as compared to start values. The significant increase of ANGPTL4 at 40 min is somewhat similar to LPL-release by tinzaparin at 40 min, which may indicate that tinzaparin could release ANGPTL4 from binding sites into the blood. ANGPTL4 is a heparin-binding protein (72) and others have shown that its concentration in blood increases after injection of heparin to normal healthy subjects and to patients with diabetes type 2 (73, 74). The release into blood makes the
ANGPTL4 amenable to be filtered out. In line with that the concentration of ANGPTL4 in blood increased less with the high flux (14%) than with the low flux filter (32%). Furthermore, in the samples of ultrafiltrates, ANGPTL4, but not ANGPTL3 was detected by the immunoassay, but the levels were relatively low.

With dialysis using local citrate there was a significant reduction in ANGPTL4 at 40 minutes and at 180 minutes compared to start value, but no significant alteration in the levels of ANGPTL3. There was a tendency for ANGPTL3 to increase after the injection of tinzaparin, but this did not reach statistical significance.

Human ANGPTL4 is a 406 amino acid residue protein with a signal peptide directing secretion that is cleaved off, an amino-terminal coiled-coil domain, a linker, and a carboxy-terminal fibrinogen-like domain (75, 76). The molecular size of ANGPTL4 is reported to be about 44-58 kDa (77). The maximal cut-off for the high flux dialyzers that were used in our study was about 15-20 kDa, but this does not exclude that there is slow passage of proteins with higher molecular mass through the filters (personal communication with manufacturer). ANGPTL4 is presumably present in plasma as monomers and oligomers, but also as fragments due to cleavage in the linker region of the protein subunit (78). Therefore HD probably eliminates mostly fragments and not necessarily intact ANGPTL4 molecules. High flux dialyzers might be favoured with regard to preservation of LPL activity, because when using these the elimination of ANGPTL4 is better than when using low flux dialyzers. A reduction of ANGPTL4, a prominent inhibitor of LPL activity, during HD may support TG break down and counteract dyslipidaemia and non-physiological distribution of free fatty acids. Both ANGPTL3 and ANGPTL4 may be considered as uraemic toxins. It is thought that malnutrition and dyslipidaemia due to dysfunction of LPL, together with the inflammation that takes place during blood membrane interaction (79),
Contribute to the risk for atherosclerosis, as part of the MIA syndrome (malnutrition, inflammation and atherosclerosis) in HD patients (80). It was hypothesized that the use of local citrate as anticoagulation during HD might prevent the intermittent disturbances of the LPL system and thereby support normal physiological energy supply to the tissues by LPL linked to the capillary endothelium. Unfortunately, HD with local citrate is in many cases complicated by clotting.
Cardiovascular disease is a major cause of mortality and morbidity in patients on chronic haemodialysis (HD). One main contributing factor is renal dyslipidaemia, characterized by an impaired catabolism of triglyceride (TG)-rich lipoproteins with accumulation of atherogenic remnant particles. The enzyme lipoprotein lipase (LPL) is a key molecule in the lipolysis of TG-rich lipoproteins. In HD patients the activity of endothelial (heparin-releasable) LPL is only half of that in age-matched, healthy controls. The present studies were performed to elucidate various conditions and factors that may have an impact on LPL-related lipid metabolism. We were able to verify, both by long term follow up and by comparison with PD patients, that the LPL-system is not exhausted by recurrent treatment with tinzaparin or UF-heparin in HD patients. However, the ability to break down the plasma TGs seems to be reduced immediately after each HD session, while this is not seen in PD patients. The amount of available LPL for release from its endothelial binding sites was similar with UF-heparin as with tinzaparin but the intermittent increase in plasma TG levels immediately after the HD session was more marked with tinzaparin than with UF-heparin. The present study also demonstrated increased levels of the assumed LPL inhibitors ANGPTL3 and 4 in plasma of HD patients. These proteins seem to accumulate as uremic toxins in patients with chronic renal disease and could further contribute to impaired LPL activity and plasma TG metabolism in these patients. We found that the ANGPTL4 in plasma can be partly reduced through the use of high flux dialyzer. Whether this improves the LPL system has to be further investigated.

PD patients have equally low pool of endothelial LPL as HD patients. This indicates that uraemia as such is likely to be the major factor that lowers the LPL activity, and that the dialysis technique is less important in this regard. However, this study indicated that PD patients have a more effective TG degradation towards the end of the dialysis session compared to HD patients. We speculate that this is because the PD patients are not exposed to anticoagulants like UF-heparin or tinzaparin and they therefore do not experience an intermittent loss of endothelial LPL activity in their tissues.

Haemodialysis with local citrate in the dialysate did not cause any release of LPL activity from endothelial binding sites to the blood circulation. In addition, ANGPTL4 was not increased.
During dialysis, in contrast to when tinzaparin was given. This type of local citrate anticoagulation may be preferable to preserve the normal function of the LPL system, but a major drawback was that in about 50% of the patients clotting occurred during the dialysis that had to be prevented by addition of other anticoagulants.

The disturbed lipid breakdown during and after each HD session may contribute to the abnormal lipid profile found in dialysis patients and may contribute to worsen the cardiovascular morbidity of these patients.
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