Uraemic sera stimulate lipolysis in human adipocytes: role of perilipin

Jonas Axelsson1, Gaby Åström2, Eva Sjölin2, Abdul Rashid Qureshi3, Silvia Lorente-Cebrián2, Peter Stenvinkel1,3 and Mikael Rydén2

1Division of Renal Medicine, Dept of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden, 2Department of Medicine (H7), Karolinska Institutet, Karolinska University Hospital, Huddinge, Stockholm and 3Division of Baxter Novum, Dept of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden

Correspondence and offprint requests to: Mikael Rydén; E-mail: mikael.ryden@ki.se

Abstract

Background. Although chronic kidney disease (CKD) is associated with dyslipidaemia and insulin resistance, the exact cause(s) are unknown. Since adipose tissue plays an important role in the development of these complications, we investigated the effect of uraemic sera on human adipocytes in vitro.

Methods. Cultured human adipocytes were incubated for 48 h with media containing sera from eight CKD Stage 5 patients or four (matched for age, sex and body mass index) healthy controls. Glycerol release (an index of lipolysis) was determined in conditioned media. RNA was isolated from the cells and quantitative polymerase chain reaction of genes involved in lipolysis was performed. In vivo lipolysis was determined by the plasma glycerol/total fat mass (from dual energy X-ray absorptiometry) ratio in 28 CKD patients and 28 matched controls.

Results. Incubation with uraemic, but not control, sera resulted in a significant (−30%) increase in spontaneous (basal) lipolysis (P < 0.05). Furthermore, uraemic but not control sera induced a selective (−30%) reduction of messenger RNA (mRNA) coding for the lipid-droplet-associated protein perilipin (PLIN) (P < 0.05), while mRNA levels of lipases, adipokines and differentiation factors did not differ between the groups after incubation. Also, consistent with our in vitro data, in vivo plasma glycerol/fat mass ratio was significantly elevated in uraemic patients as compared to controls (5.23 ± 4.1 versus 3.41 ± 2.3 μM/kg, P < 0.05).

Conclusions. Undefined circulating factors in CKD patients increase basal lipolysis in human adipocytes in vitro, probably by attenuating the expression of the lipolytic regulator PLIN. Since in vivo lipolysis is a well-established risk factor for insulin resistance and cardiovascular disease, these effects may promote increased morbidity and mortality in CKD.

Keywords: adipocytes; chronic kidney disease; human; lipolysis; perilipin

Introduction

While patients with chronic kidney disease (CKD) suffer from a significantly higher mortality than the general population [1], the exact reasons are unknown. However, the advent of decreased renal function—regardless of diabetic status—is associated with a marked increase in insulin resistance [2, 3] and manifest dyslipidaemia [4], both of which are associated with mortality in this patient group [5, 6].

Numerous recent studies in the general population demonstrate that adipose tissue is mechanistically linked to insulin resistance and type 2 diabetes mellitus [7]. Increased lipolysis in adipose tissue is thought to be an important determinant of dysmetabolism, where it is linked to increased circulating nonesterified fatty acids (NEFAs), liver dysfunction and peripheral (i.e. muscle and pancreas) lipotoxicity [7]. Furthermore, adipose tissue from obese individuals is characterized by a low-grade inflammation and infiltration of macrophages, leading to increased production of proinflammatory adipokines, including tumor necrosis factor (TNF)-α, interleukin-6 (IL-6) and Monocyte chemotactic protein-1 (MCP-1). These secreted factors promote insulin resistance via local and possibly peripheral effects [8]. In CKD, the role of adipose tissue is unclear [9], with some data even suggesting a survival advantage of obesity in patients at later stages of CKD [10]. However, other epidemiological studies suggest that uraemic fat is also associated with a proinflammatory environment [11] and a marked insulin resistance [12].

Fat cell lipids are stored in the form of triglycerides (TGs) in lipid droplets. Adipocyte lipolysis is a tightly regulated enzymatic process in which stored TGs are hydrolysed into NEFAs and glycerol. In mammals, lipolysis is mediated via three enzymes with preferential specificity for different lipid products: adipose triglyceride lipase (ATGL) hydrolyses triglycerides, hormone-sensitive lipase (HSL) preferentially uses di- and to a lesser extent triglycerides as substrates, while monoglycerides are broken down by monoglyceride lipase. However, a number of other proteins are essential in regulating lipolysis, one of
the most important being the phosphoprotein perilipin (PLIN), present on the surface of the intracellular lipid droplets. PLIN plays dual roles in lipolysis. In the basal unphosphorylated state, it inhibits lipolysis by surrounding the lipid droplets, thus preventing the access of lipases to the lipid surface. Following hormonal stimulation with β-adrenergic agonists, PLIN is phosphorylated by protein kinase A, altering its conformation and enabling it to act as an anchoring protein recruiting lipases, in particular HSL, to the droplet surface [13]. The dual role of PLIN is well reflected in the phenotype of PLIN−/− mice [14], which display an increase in basal (spontaneous) lipolysis but an almost completely abrogated hormone-stimulated lipolysis. PLIN is therefore central to adipocyte lipid turnover, and changes in PLIN expression in human WAT adipose tissue correlate well with differences in basal and stimulated lipolytic activities [15, 16]. Pro-lipolytic cytokines, such as TNF-α and IL-6, stimulate adipocyte lipolysis via mechanisms that include decreased PLIN expression [16].

Given the importance of adipocyte lipolysis in promoting insulin resistance, together with the high prevalence of insulin resistance in CKD, we designed experiments to determine whether uremic sera differentially regulates human adipocyte lipolysis as compared to control sera. For this purpose, we incubated differentiated human adipocytes with sera from human uraemic and matched healthy donors and assessed lipolysis as well as the expression of genes of importance for lipolysis and adipocyte differentiation. Finally, estimates of in vivo lipolysis were assessed in plasma from stage 5 CKD patients and matched controls.

### Materials and Methods

#### Patient cohorts

Serum was collected from eight CKD stage 5 nondiabetic patients [six males, glomerular filtration rate (GFR) 10 ± 5 mL/min, age 50 ± 10 years, body mass index (BMI) 25.7 ± 4.6 kg/m²] close to, but not yet on, dialysis. Three of the CKD patients showed signs of wasting, a condition that in cancer research has been defined as involuntary weight loss >5% of the body weight within 3 months or >10% in 6 months [17]. One was a 42-year-old male who had lost 9.5 kg (100% body weight) in the 6 months prior to inclusion, one was a 44-year-old female who had lost 6.7 kg (9% body weight) in the 3 months prior to inclusion and one was a 57-year-old male who had lost 6.5 kg (5% body weight) in the 3 months prior to inclusion. The remaining five CKD patients were weight stable prior to inclusion. Serum was also obtained from four controls (three males, GFR 103 ± 12 mL/min) matched for age (43 ± 15 years) and BMI (24.3 ± 2.6 kg/m²). Briefly, after an overnight fast, 20 mL of venous blood was drawn from each donor and cold centrifuged to obtain serum. Serum was then aliquoted into 2-mL Eppendorf tubes and stored at −80°C until use.

We also analysed plasma glycerol levels in 28 nondiabetic CKD Stage 5 patients (19 males, age 50 ± 14 years, GFR 12 ± 4 mL/min) close to, but not yet on, dialysis and 28 nondiabetic controls randomly selected from a general population material (21 males, age 53 ± 12 years, GFR > 90 mL/min). Serum was analysed for albumin and triacylglycerols. The clinical characteristics of the two groups including the concomitant medications in CKD patients are detailed in Table 1. These subjects were part of ongoing prospective cohort studies of incident dialysis patients [18] and CKD Stage 3–4 patients.

Ethical approval for the study was obtained from the Ethical Committee of the Karolinska University Hospital prior to the start of the study, and all patients gave their signed informed consent.

#### Adipocyte cultures and treatments

Subcutaneous white adipose tissue was obtained from eight (two males, age 42 ± 10 years, BMI 27.8 ± 3.0 kg/m²) otherwise healthy donors undergoing surgery for nonmalignant disorders. The stroma-vascular fraction was isolated and preadipocytes were differentiated as previously

### Table 1. The clinical characteristics of the CKD Stage 5 patients and population controls in whom plasma glycerol was measured; high sensitivity C-reactive protein

<table>
<thead>
<tr>
<th>Variable</th>
<th>CKD Stage 5 (n = 28)</th>
<th>Controls (n = 28)</th>
<th>P-value&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (% males)</td>
<td>68</td>
<td>75</td>
<td>ns</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 ± 14</td>
<td>53 ± 12</td>
<td>ns</td>
</tr>
<tr>
<td>GFR (mL/min)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 ± 4</td>
<td>96 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 4</td>
<td>25 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>S-albumin (g/L)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33 ± 6</td>
<td>40 ± 3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Haemoglobin (g/L)</td>
<td>107 ± 13</td>
<td>146 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S-triacylglycerols (mmol/L)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.98 ± 1.10</td>
<td>1.62 ± 0.89</td>
<td>ns</td>
</tr>
<tr>
<td>P-glycerol (μM)</td>
<td>88 ± 65</td>
<td>73 ± 32</td>
<td>ns</td>
</tr>
<tr>
<td>P-glycerol/FBM&lt;sup&gt;d&lt;/sup&gt; (μM/kg)</td>
<td>5.23 ± 4.1</td>
<td>3.41 ± 2.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HbA1c %</td>
<td>4.8 ± 1.1</td>
<td>5.0 ± 1.0</td>
<td>ns</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>10.0 (0.2–83.0)</td>
<td>3.4 (0.4–32.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FBM, kg</td>
<td>20.5 ± 8.6</td>
<td>25.2 ± 10</td>
<td>ns</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>50.4 ± 8.9</td>
<td>56.1 ± 9.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>β-Blockers (%)</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium channel blockers (%)</td>
<td>47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin-converting enzyme</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inhibitors/angiotensin receptor blockers (%)</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretics (%)</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate binders (%)</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythropoiesis-stimulating agents (%)</td>
<td>90</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated as the mean of urea and creatinine clearances obtained from a 24-h urine collection.

<sup>b</sup>Serum samples were obtained from the same subjects to measure circulating triacylglycerol and albumin.

<sup>c</sup>Fat body mass.

<sup>d</sup>ns= statistically non significant.
described [16]. In vitro differentiated human adipocytes at Day 10 were incubated for 48 h with Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco; Invitrogen, Carlsbad, CA), supplemented with 10 mM cortisol, 66 mM/L insulin, 1 mM/L triiodothyronine, 33 µM/L biotin, 17 µM/L pantothenate, 10 µM/L transferrin, 15 mM/L HEPES, 100 µg/mL penicillin/streptomycin and 2.5 µg/mL amphotericin B with or without the addition of serum from individual CKD patients or healthy donors. Comparisons were made against cells incubated in media with no addition of serum. Prior to use, all sera were dialysed over a 1-kDa membrane (Spectra/PorBiotech Cellulose Ester Dialysis membranes, MWCO 1 kDa) against DMEM low-glucose media (Gibco; Invitrogen) in order to remove excess salts, urea and other small-molecular-weight uremic toxins. In brief, 1–1.5 mL of serum was dialysed at +8°C against 500 mL media for a total of 48 h with one media replacement after 24 h.

The optimal concentration of serum for the incubation medium was defined through titration experiments comprising 0, 1, 5, 10 and 20% uraemic or healthy serum added to the culture medium. The 5% serum concentration was selected for all further studies since this was the highest concentration at which the adipocytes appeared fully viable and did not display any signs of apoptosis, determined by Oil Red O staining and direct microscopy. All incubations were performed in duplicates. Medium aliquots were removed and kept at −20°C for subsequent measurement of glycerol concentration (an index of lipolysis) using a sensitive bioluminescence method [19]. Samples from each well were analysed in duplicates.

Quantitative reverse transcription (RT)-polymerase chain reaction

Quantitative RT-polymerase chain reaction (PCR) was performed as described previously [20]. Total RNA was extracted using the NucleoSpin RNA II (Macherey-Nagel GmbH, Düren, Germany). RNA concentration and purity were assessed spectrophotometrically with a Nano Drop ND-1000 or with an Agilent Bioanalyzer. Half microgram total RNA was reverse transcribed to cDNA using Omniscript reverse transcription kit (Qiagen) and random hexamer primers (Invitrogen). Five nanograms of cDNA was mixed with 2× iQ SYBR Green (Bio-Rad Laboratories Inc., Hercules, CA) and TaqMan primers (Applied Biosystems) in a final volume of 20 µL. TaqMan primers and probes were used to detect IL-6 (Hs00174131_m1), TNF-α (Hs00174128_m1) and MCP-1 (CCL2; Hs00234140_m1). Quantitative real-time PCR was performed in an iCycler IQ™ (Bio-Rad Laboratories Inc., Hercules, CA). Messenger RNA (mRNA) levels were determined by a comparative threshold cycle (Ct) method. Ct values were normalized to the reference rRNA 18S, which was amplified in parallel reactions. The PCR efficiency in all runs was close to 100% and all samples were run in duplicate.

Glycerol measurements in plasma

Plasma glycerol was determined by bioluminescence [19] using heparin plasma. All samples were run in duplicates (2× 25 µL) and mean values were corrected for total fat mass (in kilograms) as previously described [17].

Statistical methods

Continuous variables are presented as mean ± SD in text and mean ± SEM in figures. Group differences were determined by Student’s paired or unpaired t-test as indicated. Chi-square test was used for nominal variables. Statistical significance was set at the level of P < 0.05. Statistical computations were carried out using standard statistical software.

Results

Incubation with uraemic sera increases basal but not stimulated lipolysis

Sera were obtained from eight CKD Stage 5 subjects and four control subjects matched for BMI, age and gender. The inclusion of 5% human serum from healthy donors had no negative effects on the gene expression and/or phenotype of adipocytes in the cultures (data not shown). Following incubation, cells exposed to healthy donor sera showed no significant changes in basal lipolysis as compared to cells treated with serum-free medium (99.5 ± 64%; Figure 1A). However, treatment with

Fig. 1. Effects of CKD and control sera on lipolysis. Sera from CKD Stage 5 (n = 8, including three subjects with wasting) and matched controls (n = 4) were obtained. Differentiated adipocytes were incubated for 48 h in regular medium supplemented with 5% of the respective donor sera. All incubations were performed in duplicates using individual serum samples and glycerol release was compared to that in cells incubated with medium alone (control cells). Glycerol levels in each well were determined in duplicates. Data are presented as box plots where the upper and lower limits represent the 90th and 10th percentile, respectively. The horizontal bar in the rectangular box represents the median, whereas the upper and lower limits of the box represent the 75th and 25th percentile, respectively. The Y-axis is in a log-transformed scale. (A) Sera from CKD subjects induced a significant increase in basal glycerol release, whereas sera from control subjects displayed levels similar to those in control cells. (B) Cells treated as in (A) were incubated with 10−6 isoprenaline for 3 h after which glycerol release was determined. Although isoprenaline induced an expected 10- to 20-fold increase in lipolysis compared to control cells, there was no significant difference between cells incubated in the two types of sera. *P < 0.05 by Student’s paired t-test.
medium containing uraemic sera resulted in a significant ~30% increase in basal lipolysis (129 ± 84%, P < 0.05; Figure 1A). While the nonselective β-adrenergic agonist isoprorenaline (10^{-6} mmol/L for 3 h) induced a significant increase in lipolysis, no difference between cells treated with sera from the two groups was observed (Figure 1B). We also performed a direct comparison between the effect of sera from the three CKD patients that fulfilled the criteria of wasting with that of sera from three weight-stable CKD patients matched for gender, age and BMI (graph not shown). In the conditioned media, there was no significant difference in absolute glycerol levels either for basal (4.75 ± 2.4 and 5.0 ± 2.5 μmol glycerol, wasted and weight stable, respectively) or stimulated (68.9 ± 21.9 and 61.5 ± 20.5 μmol glycerol, wasted and weight stable, respectively) lipolysis.

**Incubation with uraemic sera downregulates PLIN expression**

mRNA levels of PLIN and the key lipases HSL and ATGL, as well as the adipogenic transcription factor PPARγ, were analysed in adipocytes exposed to uraemic and control sera (Figure 2). While no differences between the three groups were observed in the expression of HSL, ATGL and PPARγ (Figure 2B–D), PLIN mRNA was reduced by ~30% in cells exposed to uraemic sera (73 ± 12% of control, P < 0.05; Figure 2A). This effect was not due to secondary mechanisms affecting the expression of pro-lipolytic factors in the fat cells themselves since the mRNA levels of TNF-α and IL-6 were not different between the groups (data not shown).

**Elevated in vivo lipolysis in uraemic patients compared to matched controls**

As our results demonstrated that circulating factors in uraemic sera affect basal fat cell lipolysis *in vitro*, we determined if CKD Stage 5 patients display increased *in vivo* lipolysis. We analysed plasma from 28 CKD Stage 5 patients and 28 controls with similar age, gender distribution and BMI. The clinical data including concomitant medications of these donors are detailed in Table 1. As expected, uraemic subjects had significantly lower haemoglobin values and S-albumin but higher mean C-reactive protein. Although there was a small difference in lean body mass determined by dual energy X-ray absorptiometry (DEXA), the total fat mass was not significantly different between the two groups. Plasma glycerol levels were corrected for total fat mass. Uraemic patients displayed a significantly higher glycerol/fat mass index compared to controls (5.23 ± 4.1 versus 3.41 ± 2.4 μM/kg fat mass, P < 0.05; Figure 3).

**Discussion**

In this study, we report that uraemic serum increases basal, but not hormone-stimulated, lipolysis. The clinical relevance of this finding is confirmed by the observation of increased circulating glycerol levels in CKD Stage 5 patients and offers a novel insight into the possible mechanisms of uraemic dyslipidaemia. Indeed, increased lipolysis is a hallmark of several clinical conditions associated with an increased cardiovascular disease (CVD) risk—including
Uraemic sera stimulate lipolysis

To the best of our knowledge, no previous studies have investigated the effect of uraemic serum on human adipocyte function. However, our data support a previous observational study in a limited number of patients, demonstrating higher circulating levels of NEFAs and glycerol in CKD patients [26]. However, as that study did not take into account differences in fat mass between patients and controls, our data extend their finding by demonstrating that glycerol levels are increased in CKD patients also following correction for fat mass.

While our results are relevant to understanding the dysmetabolism of uraemia, we do not identify the exact molecules in uraemic serum that mediate the pro-lipolytic effect. Nevertheless, the fact that we dialysed the sera prior to cell incubation precludes that the observed effects stem from differences in the concentrations of pH, salts (including sodium and potassium) or urea. Indeed, our study suggests that the lipolytic effect is mediated by molecules >1 kDa, plausibly retained polypeptides. In other patient groups, a number of pro-lipolytic hormones and cytokines have been identified, including TNF-α [27]. However, we find it unlikely that the effects observed in our study are due to effects from this cytokine since TNF-α affects the expression of several genes in the lipolytic cascade including PLIN but also HSL [28]. Moreover, TNF-α inhibits adipocyte differentiation by downregulating differentiation factors, such as PPARγ. Since the expression of lipases and PPARγ was unaltered in our in vitro experiments, it is very unlikely that TNF-α contributes to the observed aberrations in adipocyte function. Furthermore, although it is well known that adipocytes can produce TNF-α, the adipocyte mRNA expression of TNF-α and other inflammatory factors was not affected by incubation with uraemic sera in the present study. Of interest, Zhou et al. [29] recently reported that asymmetric dimethylarginine (ADMA), known to be elevated in CKD where it correlates with endothelial dysfunction [30], induced lipolysis and decreased perilipin in human adipocytes or their effect on PLIN mRNA expression and it is unlikely that our findings are linked to increased ADMA levels since this molecule has a molecular weight (MW) of ~200 Da and should consequently have been fully removed following the dialysis step of our serum samples. A few years ago, Fricke et al. [31] screened a peptide library from human uraemic haemofiltrate for lipolytic activity and identified two proopiomelanocortin-derived peptides (VAβ-MSH and β-MSH, MW~2.6 to 2.9 kDa) with lipolytic properties in the murine adipocyte cell line 3T3-L1. The authors did not study the effect of these peptides in human adipocytes or their effect on PLIN mRNA expression and it is not clear whether the circulating levels of these peptides are quantitatively different in plasma from age-, gender- and BMI-matched healthy subjects. Nevertheless, it would be of interest to assess the effect of these peptides in human fat cells in future studies. Atrial natriuretic peptide (MW ~3 kDa) display well-established pro-lipolytic effects in human adipocytes [32] and is present at higher circulating levels in several volume-retaining conditions, including CKD [33]. However, since ANP stimulates lipolysis via mechanisms similar to those of catecholamines (i.e phosphorylation of HSL and PLIN), we find it less likely that ANP is responsible for the observed lipolytic effect. Thus,
our results imply that unknown circulating factor(s) link decreased renal function to PLIN mRNA reduction and adipocyte dysfunction. It must be emphasized that the cells used in this study were of subcutaneous origin and we cannot exclude the possibility that qualitatively different effects could be present in visceral adipocytes. Finally, it should also be stressed that we determined lipolysis via measurements of glycerol and not by assessing NEFA levels. At the fat cell level, glycerol is a better marker of lipolysis than NEFAs since only marginal amounts of glycerol are reutilized by fat cells, whereas large portions of NEFAs are reesterified into TGs [34]. Glycerol levels in conditioned media are therefore a valid steady-state estimate, which is widely used to assess fat cell lipolysis in vitro. At the whole-body level, however, glycerol is metabolized in the liver and/or excreted by the kidneys. The most accurate method to correctly determine in vivo lipolysis is therefore to employ significantly more advanced tracer techniques using isotope-marked glycerol and NEFAs.

In conclusion, lipolysis in adipocytes derived from subcutaneous fat is increased in vitro by uraemic sera, and this is also observed in vivo. The increase in fat cell lipolysis may constitute an important pathogenic mechanism promoting the development of insulin resistance and vascular disease in CKD. Further studies using size-fractionation and proteomic analyses are needed to define the pro-lipolytic components of uraemic sera.

Acknowledgements. The results presented in this paper have not been published previously in whole or part. The present study was supported by research grants from the Karolinska institutets’ Network for Endocrinology and Metabolism (ENDOMET; to J.A.), the KI’s Diabetes Theme Center (to M.R.), the Swedish Research Council (to M.R. and P.S.), the Swedish Society for Medical Research (SSMF; to J.A.), the Swedish Diabetes Foundation, the NovoNordisk Foundation, the Swedish Cancer Society (to M.R.) and the Swedish Heart and Lung Foundation (to J.A. and P.S.). The authors would like to acknowledge the excellent technical assistance of Dr Björn Anderstam, Dr Anna Witasp, Ms. Monica Ericsson and Ms. Annika Bragfors-Hellin of the Karolinska Clinical Research Unit (KFC, Karolinska University Hospital Huddinge, Stockholm, Sweden), Ms Kerstin Wåhle at the Department of Medicine, Lipid laboratory and the help of the Research Unit (KBC) of the Karolinska University Hospital’s Department of Renal Medicine, especially Ms. Annika Nilsson, RN, Ms. Ulrika Jenseen, RN, and Ms. Annika Emmoth.

Conflict of interest statement. None declared.

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Received for publication: 7.9.10; Accepted in revised form: 18.11.10