Eur J Med Res (2007) 12: 351-355

Tubular Atrophy, Interstitial Fibrosis, and Inflammation in Type 2 Diabetic db/db Mice. An Accelerated Model of Advanced Diabetic Nephropathy

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Abstract
Objective: Advanced diabetic nephropathy (DN) is difficult to address experimentally in mice because available models of DN lack global glomerulosclerosis and major tubulointerstitial pathology. Accelerating the development of DN in mice would be desirable for feasible experimental validation of potential targets that mediate the progression to late stage DN.
Methods: 6 week old male db/db mice underwent uninephrectomy and the development of nephropathy was compared to wild-type mice and sham-operated db/db mice.
Results: Uninephrectomy at young age was associated with increased albuminuria and severe glomerulosclerosis in 37% of glomeruli at 24 weeks of age as compared to sham-operated db/db mice (8%). Uninephrectomy also increased the number of glomerular macrophages in db/db mice. The uninephrectomy-related acceleration of glomerular damage was associated with significant tubulointerstitial injury as indicated by an increase in indices of tubular cell damage, tubular dilatation, and expansion of interstitial volume. Uninephrectomy markedly increased the renal mRNA expression of Mcp-1/Ccl2, Tgf-beta, and collagen I.
Conclusion: Early uninephrectomy can accelerate the development of advanced DN in db/db mice which may be instrumental in the design of interventional studies that intend to focus on the molecular pathology of the progression to late stage DN.

Key words: kidney, diabetes, progression, mice

Introduction

Animal models remain an important tool for dissecting the molecular pathology of diabetic nephropathy DN [1]. While rodent models of DN are instrumental in studying the molecular mechanisms of early mesangial matrix remodelling and albuminuria they have been less useful in studying the molecular mechanisms that mediate the progression to late stage DN. In humans late stage DN is characterized by severe glomerulosclerosis, reduced GFR, interstitial immune cell infiltrates, tubular atrophy, and interstitial fibrosis [2]. Advanced DN develops over more than a decade in humans, hence, which remains difficult to address in animal models. In fact, all commonly used rodent models of DN lack significant tubulointerstitial lesions during a period of 6 months [1]. Experimental means that intend to accelerate the development of late stage DN in mice should enhance crucial pathomechanisms of DN, e.g. glomerular hyperfiltration [3]. Experimentally, hyperfiltration can be induced by a reduction of renal mass, in which the extent of renal mass ablation should correlate with the extent of hyperfiltration and the progression of kidney disease. We therefore hypothesized that early uninephrectomy would accelerate the progression of DN in type 2 diabetic db/db mice.

Material and Methods

Animal Studies

Male 5 week old C57BLKS db/db or C57BLKS wild-type mice were obtained from Taconic (Ry, Denmark) and kept according to the National Institutes of Health (NIH) guidelines (NIH publication no. 85–23, revised 1985). At the age of 6 weeks uninephrectomy was performed through a 1 cm flank incision under general anaesthesia with midazolam 5mg/kg and 0.05 mg/kg fentanyl in db/db and wild-type mice. For sham surgery the kidney was manipulated but not ligated. Blood and urine samples were obtained at monthly intervals for the analysis of blood glucose levels (Accu check sensor, Roche, Mannheim, Germany), urinary albumin (ELISA: Bethyl Labs, Montgomery, TX, USA), and urinary creatinine (automatic autoanalyzer: Integra 800, Roche Diagnostics, Germany). All experimental procedures had been approved by the local government authorities.

Histopathological Evaluation

From each mouse the obstructed and contralateral kidneys were fixed in 10% formalin in PBS and embedded in paraffin. Two-micrometer sections were stained with periodic acid-Schiff reagent and silver following the instructions of the supplier (Bio-Optica, s.p.a., Milano, Italy) as described [4]. Glomerular sclerotic lesions were assessed using a semiquantitative score by a blinded observer as follows: 0 = no lesion, 1 = <25% sclerotic, 2 = 25-49% sclerotic, 3 = 50-74% sclerotic, 4 = 75-100% sclerotic, respectively. 15 glomeruli were analysed per section. Quantitation of the interstitial volume was determined by superposing

a grid containing 100 (10 × 10) sampling points on photographs of 12 nonoverlapping cortical fields of silver-stained tissue (× 400) of each kidney. The number of points overlying interstitial space were counted by a blinded observer. The indices of tubular cell damage and tubular dilatation were assessed accordingly. Immunohistological staining was performed on paraffin-embedded sections using a rat anti-F4/80 (Serotec, Oxford, UK, 1:50) antibody as described [4].

Real-time Quantitative RT-PCR

Kidney segments from each animal were snap frozen in liquid nitrogen and stored at -80°C. RNA preparation and real-time RT-PCR on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) was performed as described [4]. Controls consisting of ddH2O were negative for target and the housekeeper, GAPDH. The following oligonucleotide primers (300 nM) and probes (100 n M) were used: murine collagen I-α1 (gb X 54876; bp 1984– 2102): sense, 5'-TGCTTTCTGCCCGGAAGA-3', antisense,5'-GGGATGCCATCTCGTCCA-3', internal fluorescence-labeled probe (FAM), 5'-CCAGG GTCT CCCTTGGGTCCTACATCT-3'; murine TGF-β1: forward 5'-CACAGTACAGCAAG-GTCCTTGC-3', reverse 5'-AGTAGACGATGGG-CAGTGGCT-3', FAM 5'-GCTTCGGCGTCACCGT-GCT-3'; murine CCR2: forward 5'-CCTTGG-GAAT-GAGTAACTGTGTGA-3', reverse 5'-ACAAAG-GCATAAATGACAG GATTAATG-3'; FAM: 5'-TGACAAGCACTTAGAC-CAGGCCATGCA-3'. Primers and probes for murine CCL2 and 18S rRNA were obtained as pre-developed assay reagents from PE Biosystems.

Statistical Analysis

Data are presented as mean ± SEM. Comparison of groups was performed using ANOVA and post-hoc Bonferroni`s correction was used for multiple comparisons. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Uninephrectomy and Markers of Type 2 Diabetes and DN in Male db/db mice

Technically uninephrectomy was successfully performed in 100% of mice. The surgery-related mortality was 5.6 % mainly due to bleeding complications. Uninephrectomy performed at 6 weeks of age had no significant effect on blood glucose levels of db/db mice throughout the study while there was a trend to somewhat higher body weight in uninephrectomized db/db mice (Fig. 1A and 1B). Albuminuria is the first functional marker of DN in humans and db/db mice. In fact, from 2 months of age db/db mice showed albuminuria as compared to non-albuminuric age-matched wild-type mice (Fig. 1C). Uninephrectomy was associated with higher mean levels of albuminuria at any time point except for 6 months of age (Fig. 1C). These data indicate that uninephrectomy does not affect type 2 diabetes per se but accelerates the development of albuminuria, a clinically relevant marker of DN.

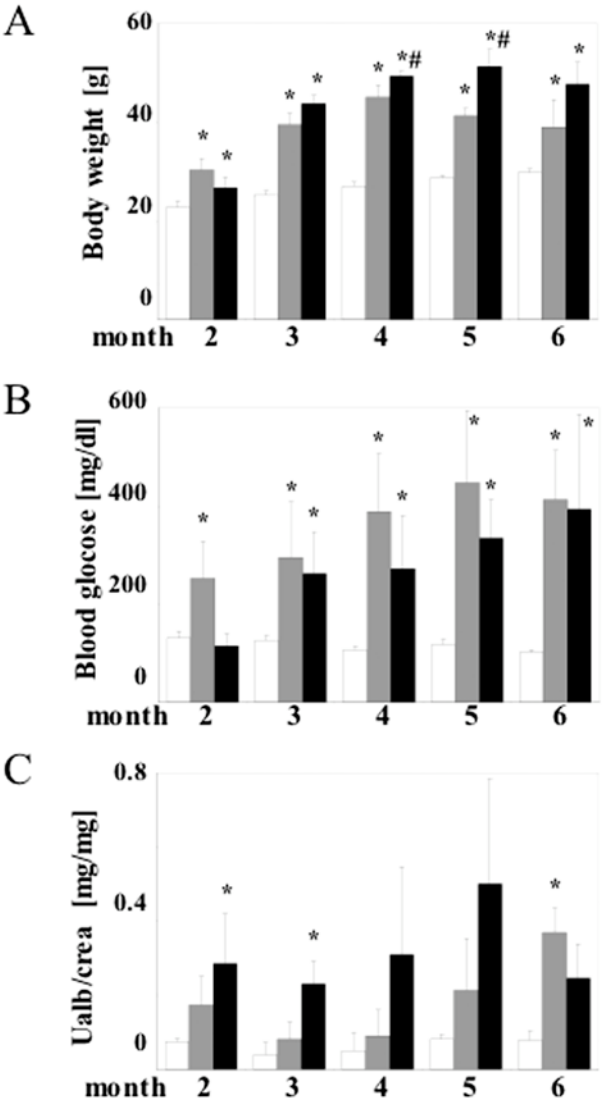

Fig. 1. Effect of uninephrectomy on body weight, blood glucose levels, and urinary albumin/creatinine ratios in db/db mice. Body weight (A), blood glucose levels (B), and urinary albumin/creatinine ratios (C) were determined at monthly intervals in wild-type mice (white barrs), sham-operated db/db mice (grey barrs), and uninephrectomized db/db mice (black barrs). Values represent means ± SEM from 6-10 mice in each group.

Uninephrectomy and Histopathological Renal Abnormalities in db/db Mice.

We next assessed the histopathological changes in kidneys of sham-operated and uninephrectomized male db/db mice.

Glomerular injury: At 6 months of age global diabetic glomerulosclerosis was more common and minor glomerular changes were less frequently observed in uninephrectomized db/db mice as compared to sham-operated db/db mice (Fig. 2A). Nephropathy in db/db

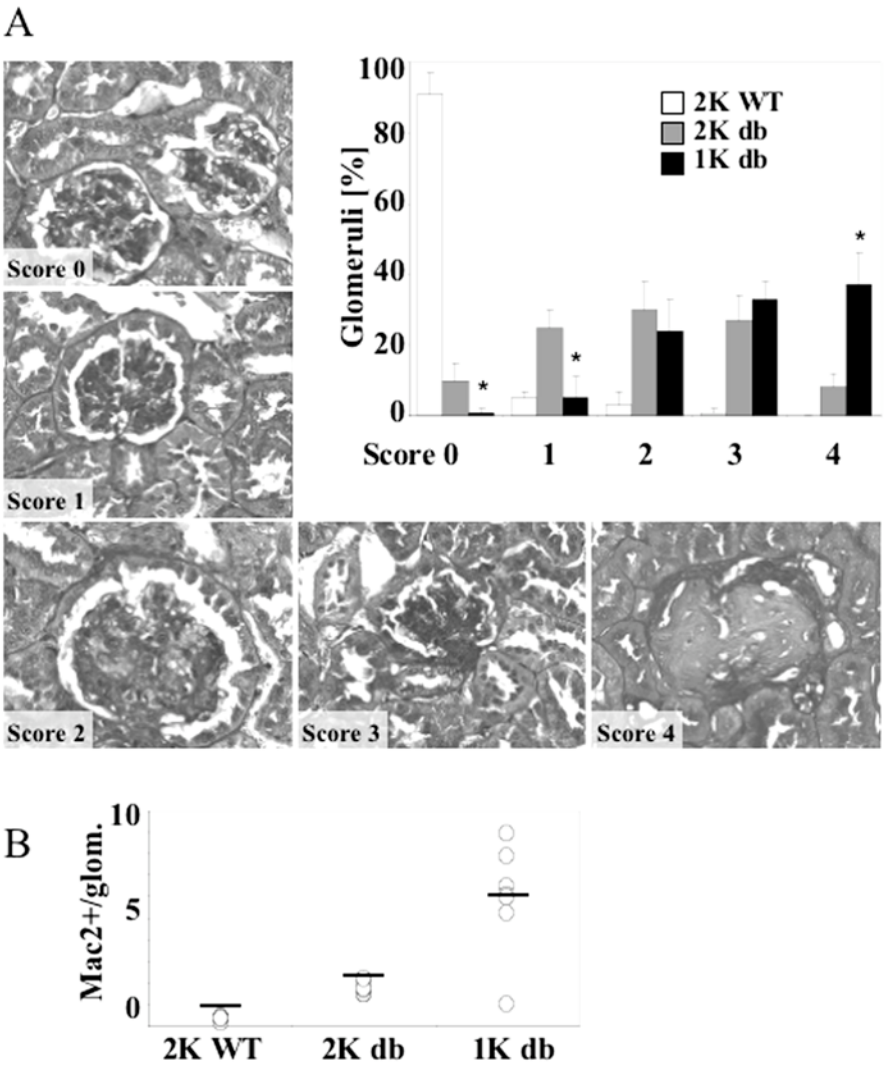

Fig. 2. Effect of uninephrectomy on glomerulosclerosis of db/db mice. A: Renal sections of the different groups of 6 months old mice were stained with periodic acid Schiff and scored for the extent of glomerulosclerosis as described in methods. Images show representative glomeruli graded to the respective scores as indicated. Note that uninephrectomy was associated with a shift towards higher scores of glomerulosclerosis, original magnification 400x. B: Renal sections were stained for Mac2 positive macrophages as described in methods. Positive cells were counted in 15 glomeruli per section. N = 7-8 in each group. 2K WT = sham-operated wild-type mice, 2K db = sham-operated db/db mice, 1K db = uninephrectomized db/db mice. * $p < 0.05$ vs. 2K WT mice, # $p < 0.05$ vs. 2K db/db mice. * $p < 0.05$ vs 1K db/db mice.

mice with type 2 diabetes is associated with glomerular macrophage infiltrates [5], however, glomerular macrophage counts were low in sham-operated db/db mice at 6 months of age (Fig. 2B). Uninephrectomy was associated with a significant increase in the numbers of glomerular macrophages ($p<0.001$ vs sham-operated db/db mice, Fig. 2B).

Tubulointerstitial injury: Tubular atrophy with flattened tubular cells, tubular dilatation, and interstitial volume are markers of tubulointerstitial damage in DN [2]. We assessed all these markers by morphometry. Tubulointerstitial injury was mild in 6 months old sham-operated db/db mice (Fig. 3). Early uninephrectomy significantly enhanced all markers of tubulointerstitial injury as compared to sham-operated db/db mice (Fig. 3).

Uninephrectomy and Renal Expression of Profibrotic and Proinflammatory Mediators

The acceleration of DN, seen with early uninephrectomy should be associated with an increase of gene expression of proinflammatory and profibrotic cytokines. We used real-time RT-PCR to quantify the mRNA expression of a number of factors that have been related to the progression of DN or chronic kidney disease. Early uninephrectomy increased the renal expression of the monocyte-chemotactic protein-1 Ccl2 and its chemokine receptor Ccr2 (Figure 4). Furthermore, uninephrectomy was associated with higher renal mRNA levels of the profibrotic cytokine tumor growth factor (Tgf)-β and collagen I-α1 in 6 months old db/db mice (Fig. 4). These data show that the histopathological and functional acceleration of DN progression after early uninephrectomy is associated with increased renal mRNA levels of proinflammatory cytokines and markers of renal fibrosis.

Discussion

Validating the role of targets for the progression to advanced DN is hampered by the lack of appropriate animal models of late stage DN. We hypothesized that early uninephrectomy would accelerate the development of severe diabetic glomerulosclerosis, albuminuria, and subsequent tubulointerstitial damage in type 2 diabetic db/db mice. Uninephrectomy was previously shown to enhance mesangial expansion in young type 2 diabetic db/db mice (6) or type 1 diabetic mice injected with streptozotocin [7]. In these studies the analysis was mostly restricted to the glomerular

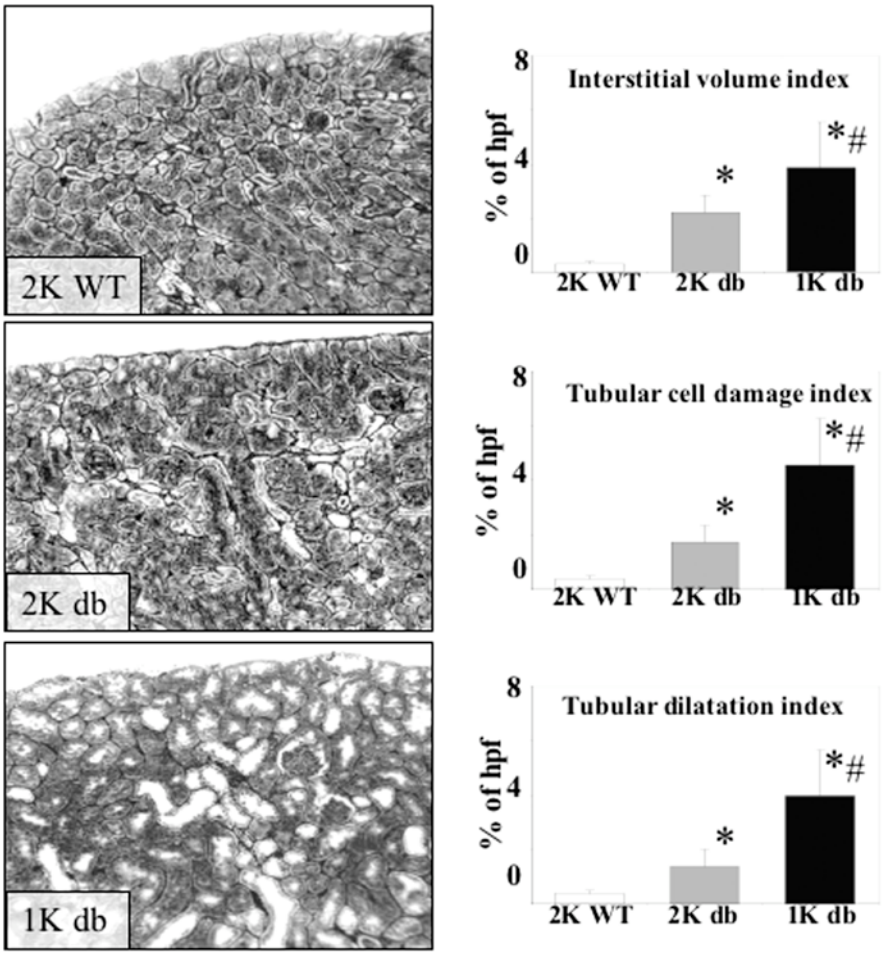

Fig. 3. Effect of uninephrectomy on tubulointerstitial injury of db/db mice. Renal sections of the different groups of 6 months old mice were stained with silver. Images show representative images from mice of each group. Original magnification 100x. Morphometry of interstitial volume, tubular cell damage, and tubular dilation was performed as described in methods. Values represent means ± SEM from 10 mice in each group. 2K WT = sham-operated wild-type mice, 2K db = sham-operated db/db mice, 1K db = uninephrectomized db/db mice. * $p < 0.05$ vs. 2K WT mice, # $p < 0.05$ vs. 2K db/db mice.

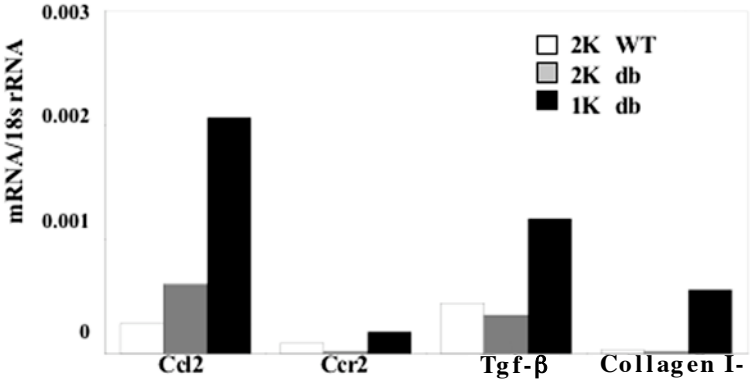

Fig. 4. Renal mRNA expression of Ccl2, Ccr2, Tgf-β, and collagen I-α1. mRNA was determined by real-time RT-PCR using total renal RNA pooled from 6 mice of each group. mRNA levels for each group of db/db mice are expressed per respective 18s rRNA expression.

pathology. Consistent with these studies we show that in type 2 diabetic db/db mice uninephrectomy performed at 6 weeks of age markedly accelerates the development of diabetic glomerulosclerosis at 6 months of age, i.e. 37% glomeruli with global sclerosis as compared to 8% in sham-operated db/db mice. This was associated with an accelerated increase in albuminuria levels. In addition to previous studies we analyzed the effect of uninephrectomy on tubulointerstitial pathology of db/db mice. Early uninephrectomy accelerated the development of tubular atrophy and interstitial fibrosis in db/db mice, consistent with the histopathological abnormalities of advanced DN in humans [2]. Interstitial macrophages are a major source of profibrotic cytokines and chemokines in late stage DN as well as in non-diabetic types of chronic kidney disease [2, 5]. The uninephrectomy-related acceleration of DN was also associated with significant interstitial macrophage infiltrates and renal expression of profibrotic factors such as Tgf-beta and chemokines such as Mcp-1/Ccl2.

Reduction of renal mass does not uniformly accelerate the progression of kidney disease in humans or mice [8]. However, a reduction of renal mass can particularly accelerate the progression of glomerulopathies probably by enhancing glomerular hyperfiltration [3, 9]. Uninephrectomy may be a prefered method of accelerating DN in db/db mice because it does not affect unrelated or other pathomechanisms of DN. Our data show that blood glucose levels and body weight were not affected by surgery. Furthermore, Cheung et al. have recently shown that a reduction of

renal mass does not affect the total food consumption, efficacy of food consumption, and the resting metabolic rate in db/db mice [10].

Together, we conclude that uninephrectomy performed at 6 weeks of age accelerates the development of advanced DN in male db/db mice. This model should be instrumental for validating targets potentially involved in the progression to late stage DN.

Acknowledgements: The work was supported by grants from the Else-Kroener-Fresenius Foundation, the EU Network of Excellence "MAIN" (FP6-502935), and the EU Integrated Project "INNOCHEM". Parts of this project were prepared as doctoral thesis at the Faculty of Medicine, University of Munich, by V. N.

REFERENCES

1. Allen TJ, Cooper ME, Lan HY. Use of genetic mouse models in the study of diabetic nephropathy. Curr Diab Rep. 2004; 4: 435-440.
2. Bohle A, Wehrmann M, Bogenschutz O, Batz C, Muller CA, Muller GA. The pathogenesis of chronic renal failure in diabetic nephropathy. Investigation of 488 cases of diabetic glomerulosclerosis. Pathol Res Pract 1991; 187: 251–259.
3. Gross ML, Amann K, Ritz E. Nephron number and renal risk in hypertension and diabetes. J Am Soc Nephrol 2005; 16 Suppl 1: S27-29.
4. Ninichuk V, Gross O, Reichel C, Khandoga A, Pawar RD, Ciubar R, Segerer S, Belemezova E, Radomska E, Luckow B, de Lema GP, Murphy PM, Gao JL, Henger A, Kretzler M, Horuk R, Weber M, Krombach F, Schlondorff D, Anders HJ: Delayed chemokine receptor 1 blockade prolongs survival in collagen 4A3-deficient mice with Alport disease. J Am Soc Nephrol 2005; 16: 977-985.
5. Chow F, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH. Macrophages in mouse type 2 diabetic nephropathy: Correlation with diabetic state and progressive renal injury. Kidney Int 2004; 65: 116 –128.
6. Bower G, Brown DM, Steffes MW, Vernier RL, Mauer SM. Studies of the glomerular mesangium and the juxtaglomerular apparatus in the genetically diabetic mouse. Lab Invest 1980; 43: 333-341.
7. Kume E, Doi C, Itagaki S, Nagashima Y, Doi K. Glomerular lesions in unilateral nephrectomized and diabetic (UN-D) mice. J Vet Med Sci. 1992; 54: 1085-90.
8. Zeier M, Geberth S, Gonzalo A, Chauveau D, Grunfeld JP, Ritz E. The effect of uninephrectomy on progression of renal failure in autosomal dominant polycystic kidney disease. J Am Soc Nephrol 1992; 3: 1119-1123.
9. Benigni A, Zoja C, Corna D, Zatelli C, Conti S, Campana M, Gagliardini E, Rottoli D, Zanchi C, Abbate M, Ledbetter S, Remuzzi G. Add-on anti-TGF-beta antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. J Am Soc Nephrol 2003; 14: 1816-1824.
10. Cheung W, Yu PX, Little BM, Cone RD, Marks DL, Mak RH. Role of leptin and melanocortin signaling in uremia-associated cachexia. J Clin Invest 2005; 115: 1659-1665.

Received: March 6, 2007 / Accepted: July 4, 2007

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