Role of the kidney for the metabolism of apolipoprotein A-IV: influence of the type of proteinuria

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Abstract

Increased plasma concentrations of apolipoprotein A-IV (apoA-IV) in chronic renal disease suggest a metabolic role of the kidney for this anti-atherogenic protein. We therefore investigated patients with various forms of proteinuria and found increased serum concentrations of apoA-IV in 124 nephrotic patients compared to 274 controls (mean 21.9±9.6 vs. 14.4±4.0mg/dl, $p<0.001$)). Decreasing creatinine clearance showed a strong association with increasing apoA-IV levels. However, serum albumin levels significantly modulated apoA-IV levels in patients with low creatinine clearance resulting in lower levels of apoA-IV in patients with low compared to those with high albumin levels (21.4±8.6 vs. 29.2±8.4mg/dl; $p=0.0007$). Furthermore, we investigated urinary apoA-IV levels in additional 66 patients with a wide variety of proteinuria and 30 controls. Especially patients with a tubular type of proteinuria had significantly higher amounts of apoA-IV in urine than those with pure glomerular type of proteinuria and controls (median 45, 14 and 0.6 ng/mg creatinine, respectively). We confirmed these results in affected members of a family with Dent's disease who are characterized by an inherited protein reabsorption defect of the proximal tubular system.

In summary, our data demonstrate that the increase of apoA-IV caused by renal impairment is significantly modulated by low levels of serum albumin as a measure for the severity of the nephrotic syndrome. From the investigation of apoA-IV in urine as well as earlier immunohistochemical studies we conclude that apoA-IV is filtered through the normal glomerulus and is subsequently mainly reabsorbed by proximal tubular cells.

Supplementary key words: apoA-IV, nephrotic syndrome, tubular proteinuria, atherosclerosis, metabolism, Dent's disease

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Introduction

Human apolipoprotein A-IV (apoA-IV) is a 46 kDa glycoprotein (1,2) synthesized in intestinal enterocytes during fat absorption and incorporated into the surface of nascent chylomicrons (3). In the fasting state the majority of apoA-IV circulates in plasma as part of a lipid-poor, small HDL-like particle that does not contain apoA-I (4). Only small amounts are lipid-free (about 4%) (5,6). In vitro studies show strong evidence that apoA-IV plays an important role in reverse cholesterol transport (7-12). Since disturbances in the reverse cholesterol transport are obvious in patients with chronic kidney disease (13,14), apoA-IV was investigated and found to be markedly elevated in hemodialysis and peritoneal dialysis patients (15-19). It was identified as a marker of kidney impairment which starts increasing in the earliest stages of kidney disease (20), and high apoA-IV concentrations predict the progression of primary non-diabetic kidney disease (21).

Patients with kidney disease present a group with an extremely high cardiovascular risk (22). Several other metabolic disturbances have been described that might contribute to this high risk (19). These include high triglyceride and Lp(a) levels, low HDL cholesterol concentrations or a disturbed lipolysis.

Severe changes in the lipoprotein metabolism have also been described in patients with proteinuria and nephrotic syndrome (23-25). The nephrotic syndrome is the consequence of increased permeability of the glomerular basement membran resulting in urinary loss of plasma proteins including albumin, transferrin, IgG, hormone binding proteins, and inhibitors of the clotting cascade like antithrombin, or protein S. Therefore nephrotic syndrome is characterized by severe proteinuria and hypoalbuminemia. In response to the subsequently decreased plasma oncotic pressure mainly the synthesis of large hepatic proteins, including apolipoproteins and lipoproteins is elevated and causes hyperlipidemia (23,24). While increased triglyceride concentrations in nephrotic patients result from decreased VLDL catabolism, the increase in LDL and Lp(a) derives from an increased synthesis (26,27). Severe proeinuria may also result from disturbed tubular re-absorption of small proteins which are physiologically secreted through the glomerula.

In recent immunohistochemical studies we observed immunoreactivity of apoA-IV in the kidney tubular cells which suggests a direct role of the human kidney in the metabolism of apoA-IV (28). To elucidate this role in more detail and by a different approach, we investigated apoA-IV serum concentrations in the present study in

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patients with various degrees and types of proteinuria. ApoA-IV serum concentrations were measured in 124 patients with a wide range of nephrotic proteinuria. In addition, serum and urinary apoA-IV were analyzed in 66 patients with glomerular and tubular type of proteinuria and a large family with Dent's disease. This disease is an X-chromosomal-linked tubular syndrome (29,30) caused by mutations in the renal chloride channel gene CLCN5. It is characterized by pronounced tubular proteinuria due to a failure in the reabsorption of low molecular size proteins by the proximal tubular system (31) and the urinary loss of these proteins.

Methods

Patients and Controls

Nephrotic syndrome. One-hundred twenty-four patients with nephrotic syndrome were recruited at the Department of Nephrology at the University of Innsbruck as part of a recently described study (25). Patients were included in the study when they underwent kidney biopsy and when they had at least one exact measurement of 24-hours proteinuria with more than 3.5 g/24 hours, serum creatinine, height and weight and a fasting blood withdrawal with collection of serum. Patients with diabetic nephropathy were excluded from the study. All patients were Caucasians and not in need of renal replacement therapy. Patients were recommended a balanced diet with daily 0.8-1 g protein per kg body weight with neither protein restricting nor protein overconsumption. Table 1 shows the clinical characteristics of patients including the histological diagnosis of the primary cause of renal disease. Patients were compared to 274 controls frequency-matched for age and sex and of the same ethnic origin without renal impairment or liver disease who were recruited in 1997 from one of the PROCAM study centers.

Proteinuric patients. For serum and urine analysis 66 patients with different forms of proteinuria were recruited at the Department of Nephrology at the University of Innsbruck and at the Feldkirch Hospital during 1999-2002. Thirty randomly selected healthy controls with comparable age and gender distribution consisted of individuals from the staff of our Department.

Dent's disease. One family with five affected male patients with Dent's disease, five female carriers and 42 unaffected family members was recruited at the Department

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of Nephrology and Dialysis at the Feldkirch Hospital. Three of the five affected patients had normal serum creatinine levels, the other two patients had already undergone a kidney transplantation. The diagnosis of Dent's disease was based on the following clinical criteria: 1) low molecular weight proteinuria; 2) hypercalciuria; and 3) the X-chromosomal mode of inheritance. All family members were analyzed for CLCN5 mutations. The affected patients carried a novel CLCN5 mutation (Lhotta et al., unpublished results). All female carriers were shown to be heterozygous for this CLCN5 mutation.

Our studies on patients with kidney impairment were approved by the institutional ethic committees and participants gave informed consent.

Laboratory procedures

Serum and EDTA plasma were taken after a 12-hour overnight fast. After low-speed centrifugation, samples were frozen and kept at -80°C prior to analysis. We calculated the creatinine clearance (CrCl) using the formula of Cockcroft and Gault (32) corrected to 1.73 m^2 body surface. Measurement of serum albumin, lipids and apoA-IV were performed in batches. Serum albumin concentrations (brom-cresol green method), total and HDL cholesterol were measured by using kits from Roche diagnostics (Basel, Switzerland). Serum apoA-IV quantification was performed with a double-antibody ELISA using an affinity-purified polyclonal rabbit anti-human apoA-IV antibody for coating and the same antibody coupled to horseradish peroxidase for detection. Plasma with a known content of apoA-IV (standardized with purified apoA-IV after phenylalanine quantification by HPLC) served as the calibration standard. Each sample was analyzed in duplicate, and intra- and interassay coefficients of variation were 2.7% and 6%, respectively. The lower detection limit of this assay was 0.002 mg/dl.

Urinary apoA-IV from patients and controls was analyzed with the ELISA described above. Immunoblot analysis of urinary apoA-IV was performed using a Novex gel system (BisTris 4-12%; 1 x MOPS; 200 V for 1h) under reducing conditions with 1:4 diluted urine, applying 10 µl per sample. A control plasma and purified apoA-IV were diluted 1:40 and 2 µl were applied to the gel. Gels were blotted for 45 minutes at 120 Volt in a cooled Hofer transphor unit. Incubation with a HRPO-labeled affinity-purified polyclonal rabbit anti-human apoA-IV antibody was followed by detection with ECL substrate (Amersham Bioscience).

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α1-microglobulin in urine was measured by nephelometry using N α1-Microglobulin Kit on the Behring NephelometryII-System (Dade Behring Marburg GmbH, Marburg, Germany). Renal proteinuria was classified as being of glomerular type if α1-microglobulin/creatinine was equal or below 14 mg/g or of tubular type if above 14 mg/g.

Statistical procedures

Statistical analysis was performed with SPSS for Windows 12.0 and SAS 9.1.3. Unadjusted comparisons of continuous variables between controls and nephrotic patients were done by unpaired t-test or by the nonparametric Wilcoxon rank sum test for not normally distributed variables such as serum triglycerides, CrCl, proteinuria, urinary protein/creatinine or apoA-IV/creatinine. Spearman correlation coefficients were computed to investigate the relationship between apoA-IV and parameters of renal dysfunction or lipid levels. The Mantel-Haenszel test of one degree of freedom was used to compare the frequency distribution of apoA-IV serum levels in categories between patients and controls. A linear model (SAS GLM procedure) was used to investigate the association of different variables with apoA-IV serum concentrations adjusting for gender without and with inclusion of interaction parameter.

Results

The anthropometric, clinical and biochemical parameters of nephrotic patients and the age- and gender-matched healthy controls are summarized in Table 1. As expected, plasma levels of albumin, total protein and creatinine as well as CrCl differed significantly between patients and controls. Patients showed a 24h-proteinuria of on average 7g.

Hyperlipidemia and nephrotic syndrome

Hyperlipidemia is one striking characteristic of the nephrotic syndrome. As shown in Table 2, all plasma lipids but HDL were significantly elevated in the nephrotic patients. Mean total and LDL cholesterol and triglyceride levels were at least 50% above the values of the control group.

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ApoA-IV and nephrotic syndrome

ApoA-IV serum concentrations were markedly increased in nephrotic patients as compared to controls (21.9±9.6 vs. 14.4±4.0 mg/dl; $p<0.001$), and the frequency distribution of apoA-IV concentrations by categories showed major differences between patients and controls (Figure 1; Mantel-Haenszel test with one degree of freedom: $p<0.001$).

In patients, serum apoA-IV levels showed the strongest correlations with serum albumin levels ($r=0.510$; $P<0.001$) (Figure 2A) and weaker correlations with proteinuria ($r=-0.304$; $p<0.001$) (Figure 2B). In addition, apoA-IV increased with decreasing renal function (Figure 2C). Generally, the correlations of apoA-IV levels with other variables, notably creatinine and CrCl, were weaker in controls than in patients (Table 3). We did not find any correlation between apoA-IV and triglyceride concentrations even if we performed the correlation analysis stratified for patients in the tertiles of triglycerides or serum albumin levels.

In order to analyze the influence of the severity of the nephrotic syndrome - as measured by serum albumin and CrCl - on apoA-IV levels, we stratified the patients according to the sex-specific medians of serum albumin (2.68 and 3.05 for women and men, respectively) and of CrCl (54 and 63 ml/min/1.73m for women and men, respectively). Figure 3A shows 5 mg/dl lower serum apoA-IV levels in case of serum albumin levels below compared to above median levels ($p=0.0014$). Nephrotic patients with CrCl below median showed about 7 mg/dl higher serum apoA-IV levels than those with CrCl above median ($p<0.0001$). In a next step, we included an interaction between CrCl groups and albumin groups, which showed borderline significance ($p=0.099$) using the significance level of 0.10 usually applied for interaction analysis. Figure 3B shows the apoA-IV levels from this analysis for the four groups (high albumin and high CrCl, low albumin and high CrCl, high albumin and low CrCl, low albumin and low CrCl). It can be seen that the difference between subjects with low or high albumin in the apoA-IV levels were small in case of high CrCl (mean±SD: 16.9±7.4 vs. 19.4±9.3 mg/dl; $p=0.25$). However, patients with low CrCl had significantly higher apoA-IV levels in case of concomitant high albumin levels when compared to those with low albumin levels (29.2±8.4 vs. 21.4±8.6 mg/dl; $p=0.0007$). It therefore seems that the known apoA-IV-increasing effect of a decreased creatinine clearance (20) is strongly modified by low albumin levels and therefore by the severity of nephrotic syndrome. When we performed the analysis

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stratified by tertiles of CrCl and tertiles of serum albumin levels, we found a consistent pattern even indicating a trend per tertile (Figure 3C). Although women have generally lower apoA-IV levels, the observed association was the same in men and women (data not shown).

When we offered 24h-proteinuria dichotomized by the sex-specific median (6.90 or 6.16 for men or women, respectively) instead of the dichotomized albumin to the model, the differences in the four groups (low proteinuria and high CrCl, high proteinuria and high CrCl, low proteinuria and low CrCl, high proteinuria and low CrCl) were similar, but less pronounced (19.0±8.5, 16.8±7.8, 28.6±9.2, 22.8±8.7 mg/dl, respectively). A smaller percentage of the variance of apoA-IV was explained by proteinuria versus albumin: with sex, albumin and CrCl in the model, 37.4% of the variance of apoA-IV was explained to which the three variables contributed 3.3%, 22.4% and 11.7%, respectively. The analogous model exchanging 24h-proteinuria for albumin explained in total only 20.0% to which the three variables contributed 3.6%, 5.1% and 11.3%, respectively.

Urinary ApoA-IV and the type of renal proteinuria

In order to analyze whether apoA-IV is lost in urine and whether the type of proteinuria (glomerular or tubular) influences the amount of apoA-IV in urine, we investigated a further sample of 66 patients with isolated glomerular (n=17) or tubular (n=49) proteinuria (Table 4) compared to 30 healthy controls. We found significantly higher apoA-IV levels in urine of proteinuric patients compared to controls (median 26 versus 0.6 ng/mg; $p<0.001$). This difference was mainly caused by significantly higher urinary apoA-IV concentrations in patients with tubular type of proteinuria compared to those with an isolated glomerular proteinuria (median 45 versus 14 ng/mg; $p<0.001$). The comparison of the three groups is presented in Figure 4A. Figure 4B shows a significant correlation of the logarithmically transformed urinary apoA-IV/creatinine with α1-microglobulin/creatinine concentrations (Spearman correlation coefficient = 0.44, $p<0.001$) demonstrating a significant association of urinary apoA-IV excretion with the intensity of tubular damage. Western blot analysis confirmed the ELISA data and the presence of intact apoA-IV and apoA-IV fragments only in urine samples from patients with a tubular component of proteinuria (Figure 4C). To find significant amounts of apoA-IV in urine patients had to have a tubular

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defect as well as a sufficient amount of apoA-IV glomerularily filtered which was no longer able to be reabsorbed by the tubular cells.

Dent's disease

Dent's disease is an X-linked renal tubular disorder which is characterized by low-molecular-weight proteinuria as one of its features (29). Its primary causes are loss-of-function mutations in the renal chloride channel gene CLCN5, that are responsible for the defective endocytic uptake of low molecular weight proteins in proximal tubular cells. To prove the proteinuric loss of apoA-IV as a tubular malfunction we measured urinary apoA-IV concentrations in a family with Dent's disease. Four out of five affected males showed elevated urinary apoA-IV concentrations compared to the five female carriers and to the non-carriers (Figure 5). Two of the patients had already undergone a kidney transplantation. One of these two patients had a normal transplant function and no apoA-IV excretion in urine as well as a normal α_1-microglobuline/creatinine level in urine. The other patient had a chronic transplant nephropathy with a serum creatinine of 2 mg/dl, α_1-microglobuline/creatinine level of 157 mg/g and an increased urinary apoA-IV/creatinine level of 50 ng/mg. Female carriers and normal individuals showed no significantly elevated urinary apoA-IV levels.

Discussion

Increased serum apoA-IV concentrations are a characteristic feature of renal disease (15-19) and are even an early marker of renal impairment (20) as well as progression of kidney disease (21). No study in humans up to now investigated apoA-IV in nephrotic syndrome. Our results show major differences between nephrotic patients and non-nephrotic patients with impaired kidney function. In a previous study we had demonstrated that apoA-IV levels were mainly explained by the loss of glomerular clearance function in patients with non-nephrotic kidney disease (20). The present study shows that apoA-IV levels in patients with nephrotic syndrome are strongly influenced by the severity of nephrotic syndrome. We used serum albumin levels as a surrogate marker for proteinuria, since this parameter is measured with a higher reliability and reproducibility than the 24-hours proteinuria. It is well known that any clearance technique which relies on urine excretion measurement is markedly influenced by substantial errors associated with 24 hour

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urine collection which is inconvenient and difficult for most patients (33). Serum albumin also provides a better insight in the metabolic situation of the disease as low albumin levels are not only the result of urinary but also of extrarenal losses, an insufficient hepatic protein production and disturbances in the distribution of albumin between the intra- and extravascular albumin pool (33). Our data clearly demonstrate that the increase of apoA-IV caused by renal impairment is significantly modulated by a low level of serum albumin. Nephrotic patients having a CrCl below the sex-specific median had about 7 mg/dl higher apoA-IV levels than those with a CrCl above the median (Figure 3A). In our previous study (20) in non-nephrotic renal disease we observed with each 11 ml/min decrease of GFR an increase of 1 mg/dl apoA-IV which is similar in size as in the present study. In nephrotic patients low serum albumin levels had a significantly apoA-IV-decreasing effect (-5 mg/dl) (Figure 3A). This was most pronounced in patients with a CrCl below the sex-specific median (-7.8 mg/dl, Figure 3B). If we repeated the regression analysis in the data from our former study (20) of non-nephrotic patients entering serum albumin into the model, we did not observe any effect of serum albumin on apoA-IV serum levels (Figure 6 provides a scatterplot of serum albumin and apoA-IV levels in these non-nephrotic patients for comparison with nephrotic patients in Figure 3A). This argues for a strong independent effect of the severity of nephrotic syndrome on apoA-IV serum levels and therefore for major differences in the metabolism of apoA-IV in nephrotic and non-nephrotic patients. The present and our previous data suggest that decreasing kidney function determined by CrCl has a strong apoA-IV level-increasing effect as long as a patient has only a non-nephrotic proteinuria. As soon as a pronounced nephrotic syndrome with its major influence on metabolic pathways develops, the disturbances in these pathways mask the effects of renal impairment to a large extent. This is most pronounced in patients with albumin levels and CrCl below the median of the patients. It is in line with calculations using a linear model in nephrotic patients which revealed that - together with sex - about 22.4% and 11.7% of the apoA-IV variance is explained by serum albumin levels or CrCl, respectively.

We hypothesize that the apoA-IV-decreasing effect in case of low serum albumin levels in severe nephrotic syndrome could either be caused by a decreased production or increased loss in the intestinum or by an increased loss of apoA-IV by glomerular filtration. A decreased production in enterocytes is conceivable considering the pronounced mucosal oedemas which can be observed in severe

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nephrotic syndrome. Furthermore, the production of apoA-IV is stimulated by leptin (34). Large amounts of leptin are lost in urine of nephrotic children although serum leptin levels remain stable (35). Since an infusion of leptin in patients with lipodystrophy with proteinuric nephropathy results in a reduction of proteinuria and hyperfiltration (36), it is conceivable that disturbances in leptin metabolism have some influence on the production of apoA-IV in the intestinum. On the other hand, experiments in experimental nephrotic rats revealed a compensatory increase in intestinal apoA-IV mRNA levels in response to the urinary loss of apoA-IV (37). ApoA-IV could also be lost into the intestinum by an intestinal hyperpermeability resulting in a leakage of enterocytic-derived apoA-IV into the intestinal lumen (38). Similar intestinal losses in patients with nephrotic syndrome have been described for albumin (39,40).

A renal loss of apoA-IV is supported by an almost sixfold relative increase of apoA-IV in urine of proteinuric patients when compared to controls. These investigations in urine present strong evidence that apoA-IV is filtered through the glomerulus and reabsorbed by the proximal tubular systems. In general, many plasma proteins are handled in this way. For example, the most abundant plasma protein, albumin with a molecular weight of about 65 kDa, shows a wide range of glomerular filtration. Various techniques used for measuring glomerular filtration revealed an amount filtered to the ultrafiltrate of 0.2 to almost 10 g/24h. Therefore, this large amount of filtered albumin has to be reabsorbed subsequently in the proximal tubular cells mostly by receptor-mediated endocytosis. The multiligand receptors megalin and cubulin are responsible for uptake of the vast majority of filtered plasma proteins including albumin in the renal tubular system (for review see reference (41).

A glomerular filtration of apoA-IV with subsquent reabsorption in the proximal tubular cells is supported by differentiating between patients with an isolated glomerular and those with an additional tubular component of proteinuria in our study. Those with a tubular component of proteinuria show an almost sixfold higher excretion of apoA-IV into urine compared to those with pure glomerular proteinuria and the amount of urinary apoA-IV correlates very well with the urinary excretion of α_1-microglobuline as a marker of tubular damage. Furthermore, it is strongly supported by data from a family with Dent's disease, an X-chromosomal-linked syndrome characterized by tubular proteinuria due to a failure of protein reabsorption

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by the proximal tubules. Affected male family members showed a pronounced increase in urinary apoA-IV which is in line with a recent proteomic approach that identified apoA-IV in higher amounts in these patients when compared to controls (42). The involvement of the proximal tubular system is in line with our recent data on apoA-IV immunoreactivity observed in healthy human renal tubular cells indicating a direct role of the human kidney for apoA-IV metabolism. ApoA-IV was predominantly found in the brush border of proximal tubules and in intracellular granules and various plasma membrane domains of both proximal and distal tubules (28). Finally, earlier data in rats demonstrated that apoA-IV is catabolized by kidney and liver and histological analysis found apoA-IV to be localized within proximal tubular cells (43). Because of its molecular weight of approximately 46 kDa, at least the lipoprotein-unbound (free) form of apoA-IV can be filtered through the glomeruli (5,7). An uptake by proximal tubular cells may then be followed by degradation, intracellular usage or even return to circulation. An at least partial intracellular usage or return to circulation by transcytosis is supported by an intact apoA-IV protein band in kidney tissue (28). A similar rescue transport in proximal tubular cells that is mediated by the receptor megalin has been described for other serum molecules such as vitamin B12 and retinol (44) as well as ApoA-I (45,46). In nephrotic syndrome apoA-IV can be largely reabsorbed as long as the nephrotic syndrome is not too pronounced and as long as no major tubular involvement of proteinuria occurs. This is in line with the results shown in Figure 3 which suggest that nephrotic patients with a CrCl below the median and low serum albumin levels and therefore a high likelihood of already having a tubular damage show a pronounced decrease in serum apoA-IV levels as compared to patients with high serum albumin levels. Due to the tubular damage they are no longer able to reabsorb the large amounts of glomerularily filtered apoA-IV and show therefore relatively low apoA-IV serum levels despite pronounced impairment of kidney function.

Our results are in line with findings in rats with puromycin-induced nephrotic syndrome (37). Those animals showed besides a pronounced decrease in serum albumin levels also a strong decrease in serum apoA-IV levels despite a significant increase in mRNA levels in the jejunum and ileum. The authors suggested that the decrease of serum apoA-IV levels was caused by urinary loss of apoA-IV. Due to the severity of the disease it is conceivable that the tubular reabsorption system for apoA-IV was no longer intact resulting in decreased apoA-IV serum levels.

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Limitations of the study

When we take into account the small absolute amount of measured apoA-IV in urine, this can not explain the large apoA-IV-decreasing effect in patients with severe nephrotic syndrome as observed in the linear model. We suspect that our ELISA systematically underestimates the absolute amount of urinary apoA-IV, for example by missing apoA-IV fragments, which we observed in immunoblot analysis of urine apoA-IV. Furthermore, a semi-quantitative comparison of an apoA-IV standard and urinary apoA-IV in immunoblots reveals that a much higher amount of apoA-IV is excreted with the urine than measured with our apoA-IV ELISA. Although we applied only up to 0.3 ng of urinary apoA-IV measured by ELISA to the gel, we found bands of similar intensity as serum samples or apoA-IV preparations from which we applied up to 20 ng according to ELISA measurements (Figure 4C).

In summary, our data demonstrate that the increase of apoA-IV caused by renal impairment is significantly modulated by a low level of serum albumin as a measure for the severity of the nephrotic syndrome. From the investigation of apoA-IV in urine of patients with various forms of proteinuria as well as from our previous immunohistochemical studies we conclude that glomerularily filtered apoA-IV is mainly reabsorbed via the renal tubular system.

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Figure legends

Figure 1: Frequency distribution of apolipoprotein A-IV (apoA-IV) serum concentrations by categories in 124 nephrotic patients and 227 controls. Mantel-Haenszel test: χ^2=89, df=1, p<0.00001.

Figure 2A-C: Correlation of serum apolipoprotein A-IV concentrations with serum albumin (panel A), proteinuria (panel B) and creatinine clearance (panel C). Correlations coefficients (r) are calculated according to Spearman.

Figure 3, Panel A and B: Influence of albumin status and creatinine clearance (CrCl) status on ApoA-IV serum levels in the nephrotic patients (n=124). Bars provide the mean (±SE) apoA-IV levels adjusted for sex, albumin status and CrCl status. Albumin status and CrCl status were defined by using the sex-specific medians of albumin (2.68 or 3.05 for women or men, respectively) and CrCl (54 or 63 ml/min/1.73m for women or men, respectively) as cutpoint. **Panel C:** Analysis similar as in Panel B but stratified by tertiles of CrCl and tertiles of serum albumin levels indicating a trend per tertile.

Figure 4: Panel A: Urinary concentrations of apoA-IV/creatinine in patients with isolated glomerular (n=17) or a glomerulartubular or tubular type (n=49) of proteinuria and 30 healthy controls. The horizontal lines represent the median concentration of each group. **Panel B:** Correlation of ln-transformed urinary apoA-IV/creatinine with α1-microglobulin/creatinine concentrations in patients with the two forms of proteinuria (r = correlation coefficient according to Spearman). **Panel C:** Western blot of urinary apoA-IV. Lane Pl. shows the apoA-IV pattern from plasma (10 ng applied to the gel), lane ST shows an affinity-purified plasma apoA-IV standard (20 ng applied to the gel), lanes C1 and C2 represent samples from controls and P1 to P8 from patients with various amounts of proteinuria (up to 0.3 ng applied to the gel). The amount of apoA-IV/creatinine, α1-microglobulin/creatinine and proteinuria/creatinine is provided for each sample. The amount of applied apoA-IV was calculated from ELISA measurements. The apoA-IV antibody could detect only one protein band in the plasma sample (lane 1), but a double band in the apoA-IV preparation (lane 2). Peptide sequencing in an earlier project confirmed that both protein bands of the apoA-IV preparation represent apoA-IV. The second, smaller

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apoA-IV isoform might therefore result from deglycosylation or proteolytic degradation.

Figure 5: Urinary concentration of apoA-IV per creatinine in a family with Dent's disease. Comparison of affected males, female carriers and healthy family members. Two affected males had already undergone a renal transplantation. One of these two patients had a normal transplant function and no apoA-IV excretion in urine as well as a normal α_1-microglobuline/creatinine level in urine (patient indicated as RTX). The other patient had a chronic transplant nephropathy (CTN) with a serum creatinine of 2 mg/dl, α_1-microglobuline/creatinine level of 157 mg/g and an increased urinary apoA-IV/creatinine level of 50 ng/mg (patient indicated as RTX+CTN).

Figure 6: Correlation of serum apolipoprotein A-IV concentrations with serum albumin in 227 patients with non-nephrotic primary kidney disease of our previous study (20). The correlations coefficients (r) is calculated according to Spearman. To describe the patient population briefly, we included Caucasian patients aged 19-65 years who had visited the outpatient department at least once during the preceding year. Exclusion criteria were serum creatinine >6 mg/dL, diabetes mellitus, malignancy, liver, thyroid or infectious disease at the time of recruitment, nephrotic syndrome defined as daily proteinuria >3.5 g/1.73m^2, organ transplantation, allergy against ionic contrast media, and pregnancy.

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Table 1: Anthropometric and biochemical data of patients with nephrotic syndrome and age- and gender-matched controls [a]

	Controls (n = 274)	Patients (n = 124)
Age	44.6 ± 12.5	44.6 ± 16.2
Gender (female/male, n, [% female])	97/177 [35.4%]	44/80 [35.5%]
BMI	26.1 ± 3.7	25.2 ± 4.2 [b]
Total protein (g/dl)	7.0 ± 0.4	5.9 ± 1.1 [c]
Albumin (g/dl)	4.9 ± 0.5	2.9 ± 0.9 [c]
Creatinine (mg/dl)	1.0 ± 0.2	1.9 ± 1.4 [c]
Creatinine Clearance (ml/min/1.73m^2)	89 ± 22	65 ± 35 [c]
	[77, 88, 98]	[34, 62, 90]
Proteinuria (g/24h/1,73 m^3)		7.0 ± 3.5
		[4.4, 5.7, 8.7]
Primary cause of renal disease		
Membranous glomerulonephritis		30 (24.2%)
Minimal change nephropathy		21 (16.9%)
IgA nephropathy		18 (14.5%)
Focal segmental glomerulosclerosis		17 (13.7%)
Membranoproliferative glomerulonephritis		9 (7.3%)
Amyloidosis		7 (5.6%)
Lupus Nephritis		7 (5.6%)
Nephrosclerosis		4 (3.2%)
Crescentic glomerulonephritis		2 (1.6%)
Others		9 (7.2%)

[a] Data are mean ± SD and [25th, 50th,75th percentile] where appropriate.

[b] $p < 0.05$, [c] $p < 0.001$ for comparison with controls.

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Table 2: Lipids and apoA-IV serum concentrations in patients with nephrotic syndrome and age- and gender-matched controls [a]

	Controls (n = 274)	Patients (n = 124)
Apolipoprotein A-IV (mg/dl)	14.4 ± 4.0	21.9 ± 9.6[b]
Total cholesterol (mg/dl)	203 ± 42	306 ± 92[b]
HDL cholesterol (mg/dl)	44 ± 13	42 ± 17
LDL cholesterol (mg/dl)	132 ± 37	214 ± 84[b]
Triglycerides (mg/dl)	136 ± 92	252 ± 194[c]
	[81, 112, 168]	[139, 210, 290]
Cholesterol / HDL ratio	4.9 ± 1.6	8.4 ± 5.0[b]

[a] Data are mean ± SD and [25th, 50th,75th percentile] where appropriate.

[b] $p < 0.001$ for comparison with controls (unpaired t-test).

[c] $p < 0.001$ (nonparametric Wilcoxon rank-sum test).

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Table 3: Bivariate Spearman correlation coefficients of plasma apolipoprotein A-IV with anthropometric, biochemical and lipid parameters.

	Controls (n = 274)	**Patients (n = 124)**
Albumin	0.088	0.510[c]
Creatinine Clearance	-0.154 [a]	-0.367[c]
Creatinine	0.148[a]	0.420[c]
Proteinuria	/	-0.304[b]
Gender	0.169[a]	0.203[a]
LDL cholesterol	0.047	-0.191[a]
HDL cholesterol	0.113	0.166
Total cholesterol	0.116	-0.124
Triglycerides	0.086	-0.002
Age	0.106	0.061

[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$

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Table 4: Comparison of serum and urinary parameters between patients with only glomerular and a tubular type of proteinuria

	Glomerular (n=17)	Tubular (n=49)
Serum		
Apolipoprotein A-IV (mg/dl)	21.7 ± 7.7	20.6 ±12.5 [a]
Urine		
Urinary protein/creatinine (g/g)	4.2 ± 3.7 [1.4, 3.0, 5.2]	10.4 ± 10.3 [b] [2.8, 6.4, 13.7]
Apolipoprotein A-IV/creatinine (ng/mg)	21 ± 24 [2, 14, 37]	118 ± 169 [c] [5, 45, 167]

[a] Serum apolipoprotein A-IV measurements were only available in 14 out of 17 patients with glomerular-tublar type of proteinuria and in 34 out of 49 patients with a tubular component of proteinuria.

[b] p<0.001, [b] p=0.035

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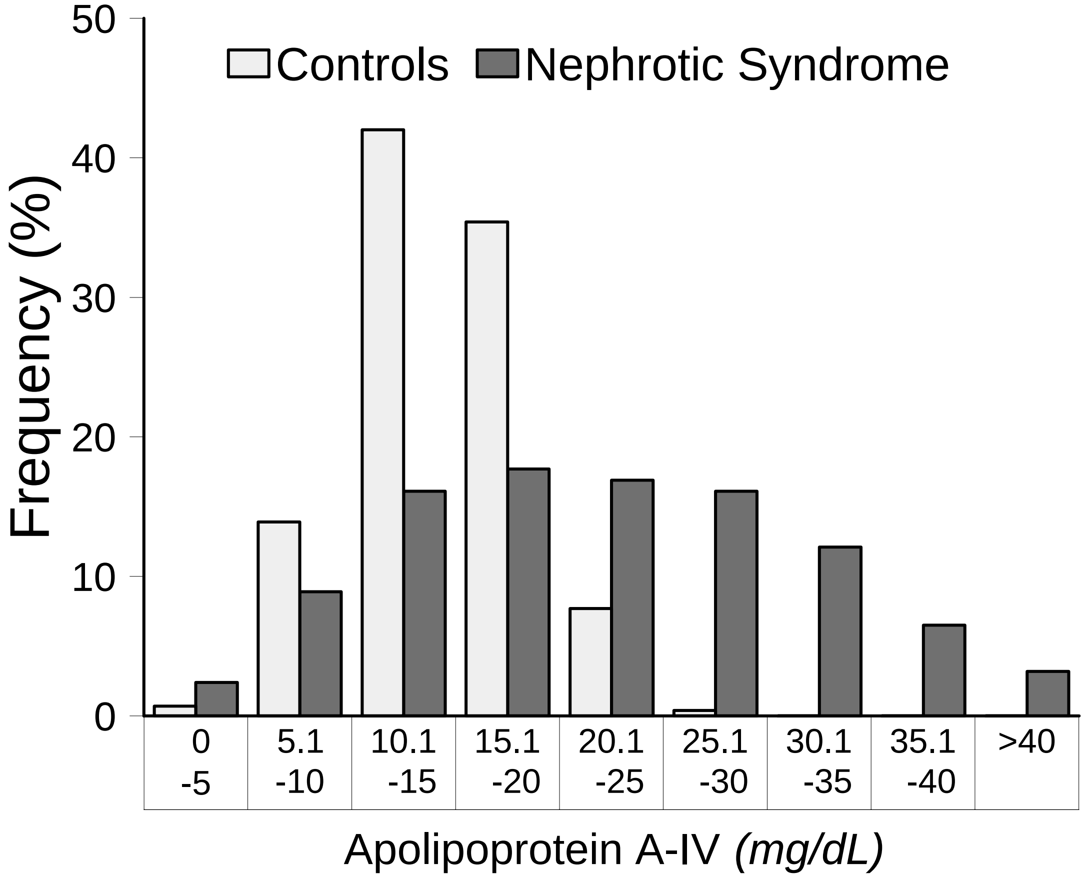

Lingenhel et al. Figure 1

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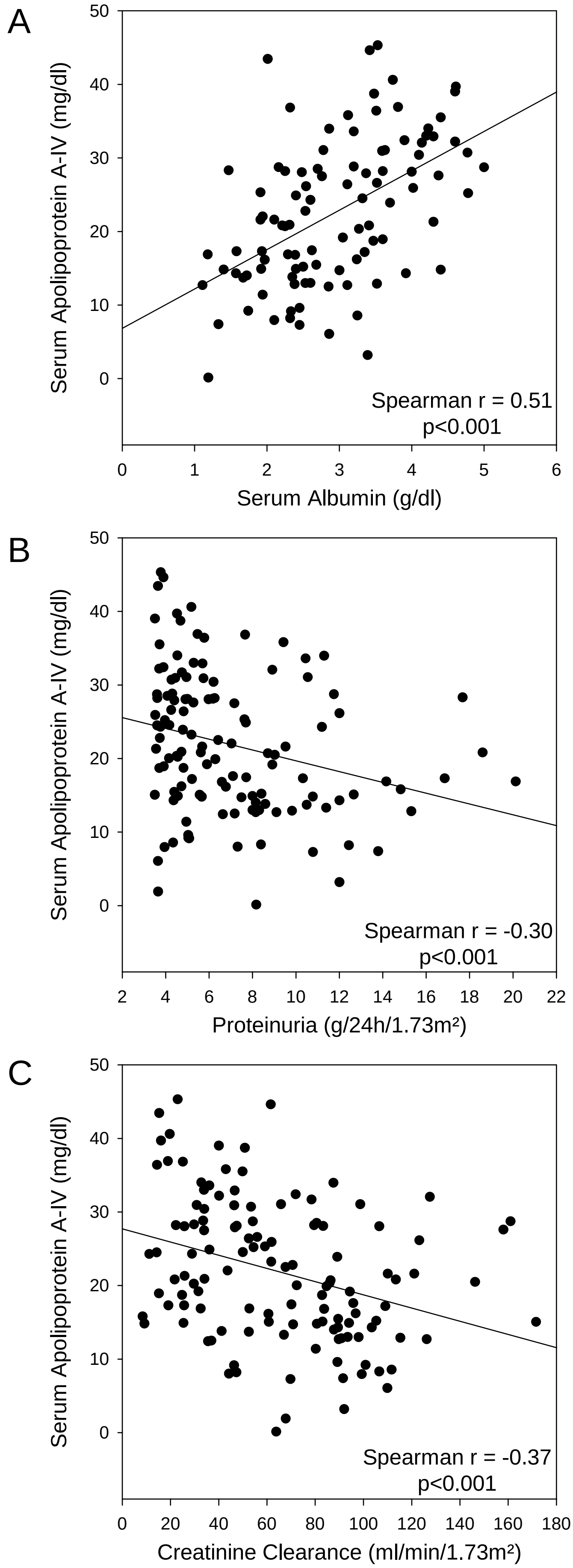

Lingenhel et al. Figure 2

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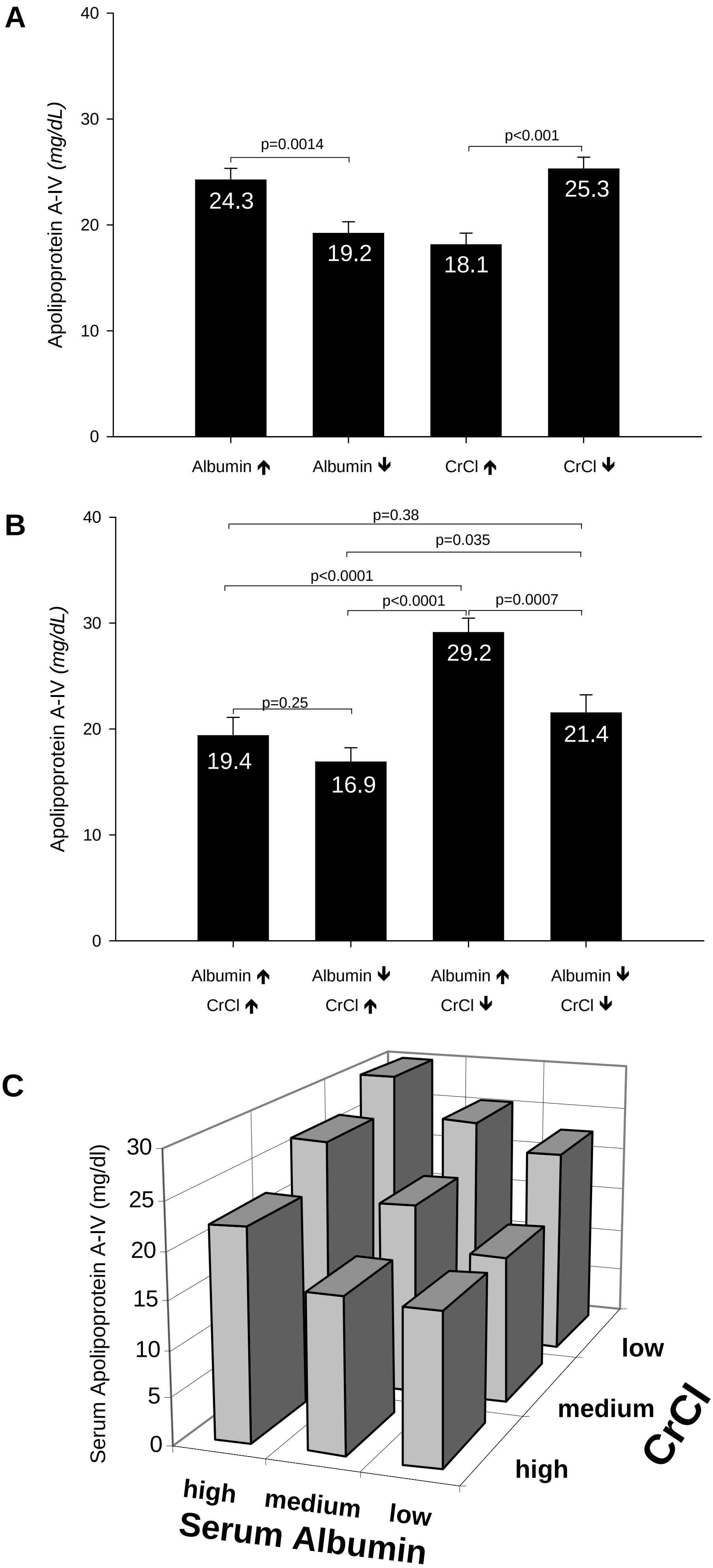

Lingenhel et al. Figure 3

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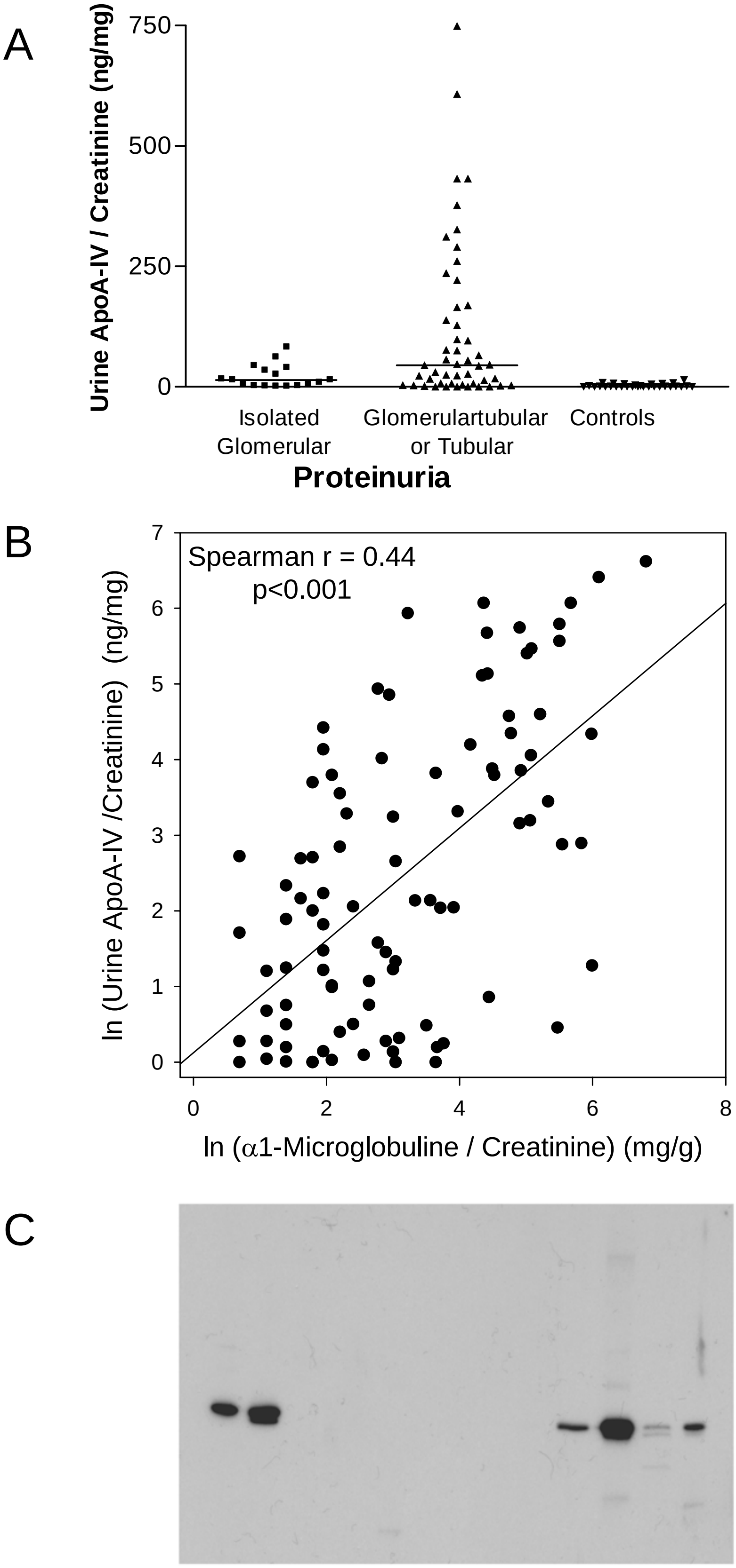

Sample	Pl.	ST	C1	C2	P1	P2	P3	P4	P5	P6	P7	P8
ApoA-IV/Cr, ng/mg			1.1	8.3	0.3	7.5	13	44	57	749	47	165
α_1-MG/Cr, mg/g			4	7	18	35	21	92	159	894	89	77
Proteinuria/Cr, g/g			-	-	5.2	6.3	3.0	0.6	0.4	7.0	0.2	0.5

Lingenhel et al. Figure 4

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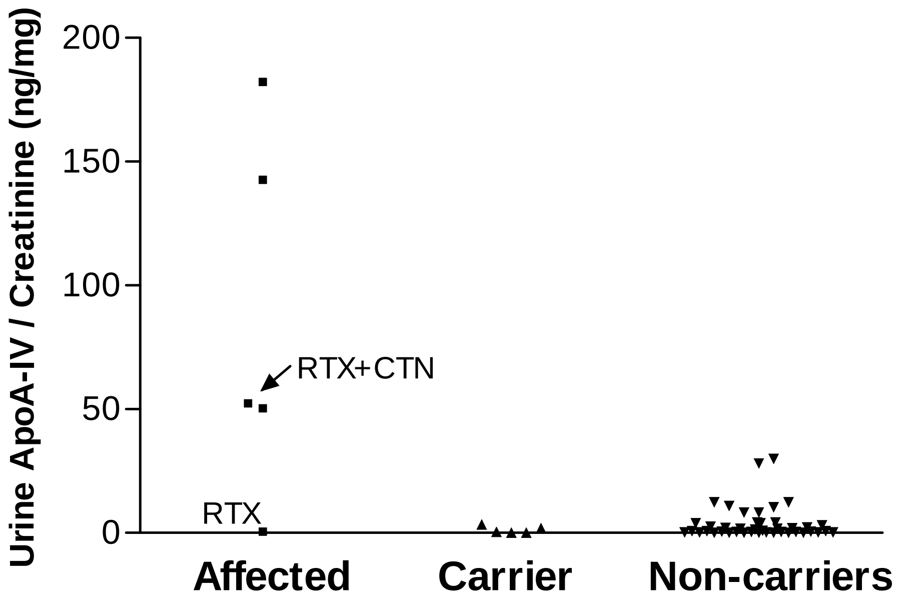

Lingenhel et al. Figure 5

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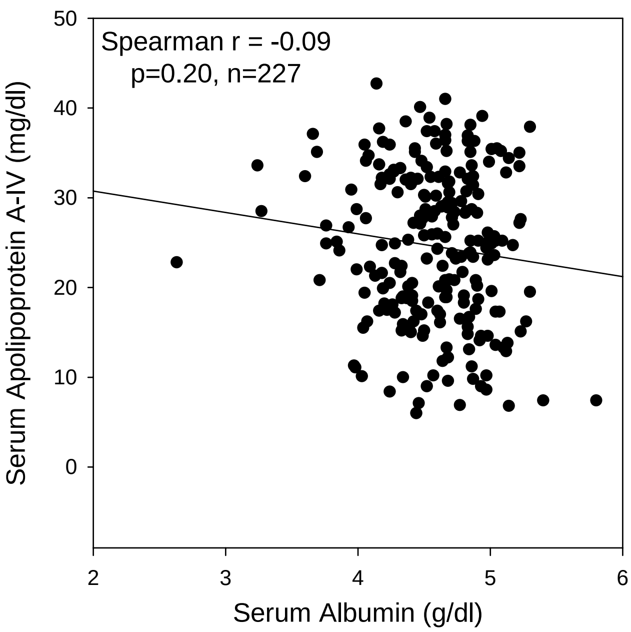

Lingenhel et al. Figure 6

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