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Alterations of retinol-binding protein 4 species in patients with different stages of chronic kidney disease and their relation to lipid parameters

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ABSTRACT

Retinol-binding protein 4 (RBP4) is elevated in patients with chronic kidney disease (CKD) and has been discussed as marker of kidney function. In addition to an elevated concentration, the existence of truncated RBP4 species, RBP4-L (truncated at last C-terminal leucine) and RBP4-LL (truncated at both C-terminal leucines), has been reported in serum of hemodialysis patients. Since little is known about the occurrence of RBP4 species during the progression of CKD it was the aim of this study to analyse this possible association. The presence of RBP4, RBP4-L, RBP4-LL and transthyretin (TTR) was assessed in serum of 45 healthy controls and 52 patients with stage 2–5 of CKD using ELISA and RBP4 immunoprecipitation with subsequent MALDI-TOF-MS analysis. A reduction of glomerular filtration rate was accompanied by a gradual elevation of RBP4 serum levels and relative amounts of RBP4-LL. Correlation analysis revealed a strong association of the RBP4-TTR ratio with parameters of lipid metabolism and with diabetes-related factors. In conclusion, RBP4 serum concentration and the appearance of RBP4-LL seem to be influenced by kidney function. Furthermore, the RBP4-TTR ratio may provide diagnostic potential with regard to metabolic complications in CKD patients.

Introduction

The retinol-binding protein 4 (RBP4) has been supposed as new adipokine, possibly contributing to the onset of insulin resistance and type 2 diabetes mellitus in obese subjects [1,2], but mainly being influenced by kidney function [3–5].

The main function of RBP4 is the transport of retinol (ROH) to its target tissues [6,7]. To prevent the renal loss of the low-molecular weight RBP4–ROH complex (approximately 21 kDa), the transport is furthermore facilitated by the binding of transthyretin (TTR) a visceral protein with an approximate molecular weight of 55 kDa [6,8,9]. After the delivery of ROH to its target tissues, the affinity of RBP4 to TTR decreases and RBP4 is subjected to renal degradation [6,7]. Since the kidneys are the main site of RBP4 catabolism, RBP4 serum concentration is associated with renal function and has therefore been supposed as surrogate marker of kidney function [10–14]. Beside changes in the RBP4 serum concentration, changes in the appearance of RBP4 species have been described for patients undergoing hemodialysis [15,16]. For these patients increased amounts of RBP4 species have been described. Thereby the RBP4 species are characterized by a truncation at the last (further on referred to as RBP4-L) or at both C-terminal leucines (RBP4-LL) [15]. However, little is known about the importance of truncated RBP4 species in progression of chronic kidney disease (CKD). Therefore, the aim of the present study was to investigate the relative amounts of truncated RBP4 species next to RBP4 serum concentration in the progression of kidney dysfunction, namely stages 2–5 of CKD.

Materials and methods

Subjects. Serum samples of 45 subjects (27 male/18 female) without any signs of kidney disease served as controls and were obtained from the Department of Clinical Nutrition, German Institute of Human Nutrition, Nuthetal and from the Department of Endocrinology, Diabetes and Nutrition, Charité Campus Benjamin Franklin, Berlin. Additionally, serum samples of 52 patients (34

Abbreviations: CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; ESRF, end-stage renal failure; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; RBP4, retinol-binding protein 4; ROH, retinol; TTR, transtyretin.

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male/18 female) with diagnosis of CKD were obtained from the Medizinische Klinik Nephrologie, Charité Campus Benjamin Franklin, Berlin. Definition of CKD was based on kidney biopsy or reduced estimated glomerular filtration rate (eGFR <60 mL/min/1.73 m^2) for more than 3 months, proteinuria, abnormalities on the urine dipstick or sediment examination, or abnormal renal imaging studies. Renal function was quantified by eGFR which was calculated according to Modification of Diet in Renal Disease formula including serum creatinine concentration, age and gender [17]. The stages of CKD were used as grouping characteristic and were assigned in accordance to the K/DOQI guidelines [18].

Exclusion criteria were age <18 years and pregnancy. Since insulin resistance and diabetes mellitus are typical features in CKD patients, we decided to include diabetic patients in the control group. However, these diabetic patients did not show abnormalities with regard to kidney function.

The study protocol was approved by Ethics Committees of the hospitals and the University of Potsdam. Written informed consent was obtained from each subject. All anthropometric, clinical, and biochemical parameters of the participants were collected by trained personnel.

Blood was collected from an antecubital vein, immediately centrifuged, and serum was immediately frozen at −80 °C until measurement.

Measurement of RBP4 and TTR. The serum concentrations of RBP4 and TTR were quantified by non-commercial enzyme-linked immunosorband assays (ELISA) as described in detail previously [5,19]. For detection of both, RBP4 and TTR, a standard was used containing RBP4 and TTR obtained from human blood (N Protein Standard/SL OQIM, Dade Behring GmbH, Germany).

Immunoprecipitation of RBP4 and subsequent analysis by MALDI-TOF-MS. Immunoprecipitation of RBP4 was performed as described elsewhere in detail [20]. Immunoprecipitates were subsequently analyzed by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) as previously described [20]. Representative MALDI-TOF-MS spectra are depicted in Fig. 1.

The following RBP4 variants were identified using MALDI-TOF-MS analysis: non-truncated RBP4 with a median molecular weight of 21,063 Da (interquartile range = 28.3 Da), RBP4-L with a molecular weight of 20,953 Da (interquartile range = 31.4 Da) and RBP4-LL with a molecular weight of 20,837 Da (interquartile range = 21.9 Da) [21].

Data analysis. The results were expressed as medians and ranges. The amounts of the truncated RBP4-forms RBP4-L and RBP4-LL were expressed as per cent of peak height of non-truncated RBP4. The peak heights were determined in a valley-to-valley procedure. Statistical analysis was accomplished by use of non-parametric procedure (SPSS version 15.0, SPSS Inc., Chicago, USA). The Kruskal–Wallis test was used to test for significant differences between the groups. When there was a significant effect, Mann–Whitney *U*-rank test was performed to evaluate differences in proportions between cases and control subjects. Spearman–Rho was used to calculate correlation coefficients. p values of <0.05 were considered to be statistically significant (two-tailed).

Results

Subject characteristics

The characteristics of the study population are given in Table 1. There was a steady decline of eGFR from control to CKD 5 with $p < 0.001$ between all groups. Additionally, other parameters also changed with progression of kidney disease in comparison to controls: systolic blood pressure was elevated in the groups CKD 2, CKD 3 and CKD 5 ($p < 0.05$ for all), total cholesterol levels were lower in all CKD groups ($p < 0.05$ for all), LDL-cholesterol was reduced in CKD 2 ($p < 0.05$), CKD 4 ($p < 0.01$), and CKD 5 ($p < 0.01$), triacylglycerol levels were elevated in CKD 5 ($p < 0.01$), HbA1c was significantly higher in CKD 2 ($p < 0.01$), CKD 3 ($p < 0.01$) and CKD 4 ($p < 0.05$) and borderline elevated in CKD 5 ($p = 0.052$) and haemoglobin was reduced in CKD 3, CKD 4 and CKD 5 ($p < 0.001$ for all) in comparison to control subjects. Furthermore, HDL-cholesterol was lowest in the group CKD 5 in comparison to CKD 3 and CKD 4 ($p < 0.01$ for both) and in comparison to the control group ($p < 0.001$), but there were no differences to CKD 2. Moreover, BMI was higher in CKD 2 and 4 in comparison to control subjects and CKD 5 ($p < 0.05$), but there was no difference to CKD 3.

The subjects of all five groups did not differ in age, diastolic blood pressure, fasting glucose, and total serum protein concentration. Approximately 22% of the CKD patients and 13% of the control subjects were positively diagnosed for diabetes mellitus (Table 1).

RBP4, TTR, and relative amounts of RBP4 species

The RBP4 serum concentration as well as the appearance of RBP4 species changed with progression of CKD (Table 2). RBP4 serum levels were elevated in CKD 4 and 5 in comparison to control and CKD 2 ($p < 0.01$ for both). The relative amount of RBP4-LL

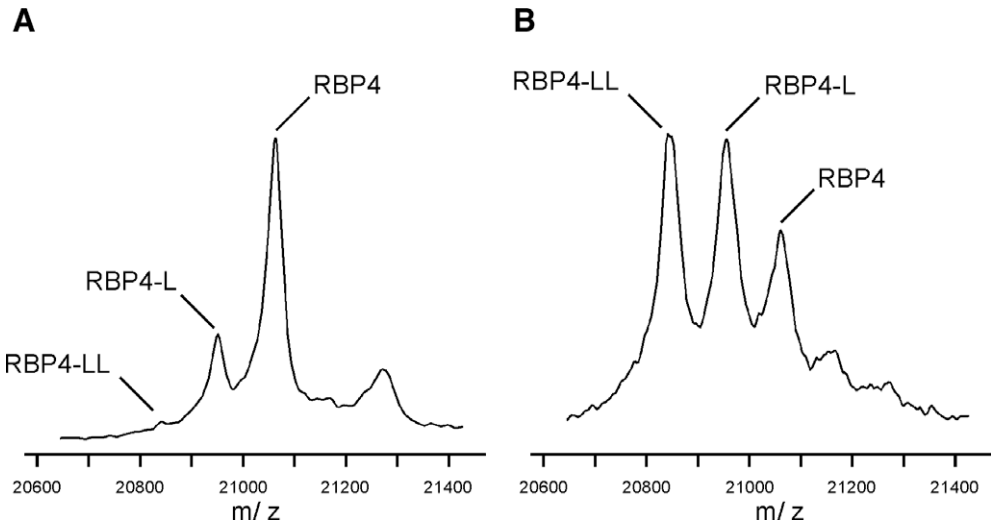

Fig. 1. Representative MALDI-TOF-MS spectra for RBP4 pattern in control subject (A) and in patients with chronic kidney disease (B). Abbreviations used: RBP4, retinol-binding protein 4; RBP4-L, RBP4 truncated at the last C-terminal leucine; RBP4-LL, RBP4 truncated at both C-terminal leucines.

Table 1
Anthropometric, clinical and biochemical data of the study population.[a]

	Control	CKD 2	CKD 3	CKD 4	CKD 5
N (m/f)	45 (27/18)	8 (8/0)	22 (12/10)	10 (6/4)	12 (8/4)
Age (years)	57 (46–71)	63 (40–73)	67 (22–73)	64 (43–75)	60 (45–72)
BMI (kg/m^2)	25.2^a (20.6–32.6)	28.3^b (23.4–35.6)	25.9^{ab} (14.8–43.4)	27.8^b (19.2–39.1)	24.5^a (17.0–27.4)
SBP (mm Hg)	123^a (100–146)	132^b (116–160)	136^b (88–198)	138^{ab} (90–156)	156^b (97–182)
DBP (mm Hg)	77 (57–95)	83 (64–87)	73 (46–116)	67 (42–107)	89 (54–106)
Total cholesterol (mg/dL)	211.2^a (132.0–325.8)	160.2^b (100.4–239.4)	189.2^b (81.1–321.0)	158.0^b (119.7–274.1)	158.3^b (69.5–196.9)
HDL-cholesterol (mg/dL)	56.4^a (31.7–109.7)	40.2^{bc} (34.7–88.8)	54.1^{ac} (27.0–104.3)	44.6^{ac} (23.2–96.5)	34.7^b (23.2–46.3)
LDL-cholesterol (mg/dL)	132.4^a (63.7–237.8)	106.8^b (58.7–169.1)	115.8^{ab} (42.7–218.2)	87.0^b (71.3–216.2)	106.2^b (42.1–123.6)
Triacylglycerides (mg/dL)	100.9^a (45.1–325.7)	131.1^{ab} (82.5–260.0)	157.9^{ab} (43.9–639.0)	134.3^{ab} (43.9–561.4)	145.6^b (54.4–377.2)
Diabetic patients (%)	13.3	0	22.7	50	16.6
Fasting glucose (mg/dL)	89.2 (63.7–121.7)	117.1 (78.2–150.9)	83.3 (62.0–380.0)	97.3 (73.0–390.9)	93.6 (67.3–136.4)
HbA1c (%)	5.4^a (4.6–6.3)	5.9^b (5.4–6.4)	5.9^b (4.2–8.7)	6.6^b (4.7–8.8)	6.2^{ab} (4.5–6.9)
eGFR (mL/min/1.73 m^2)	81^a (60–116)	67^b (60–73)	44^c (30–56)	25^d (15–29)	9^e (3–15)
Hemoglobin (g/dL)	14.0^a (11.8–18.8)	14.9^a (11.5–18.2)	12.7^b (7.7–15.7)	11.4^{bc} (8.5–12.9)	9.8^c (6.8–14.0)
Total serum protein (g/dL)	6.7 (6.1–7.1)	7.2 (6.4–9.0)	6.7 (5.4–8.1)	6.8 (0.6–7.7)	6.3 (4.6–7.2)

[a] All data are presented as median and range. Abbreviations used: BMI, body mass index; CKD 2–5, chronic kidney disease stage 2–5; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL, high density lipoprotein; LDL, low density lipoprotein; SBP, systolic blood pressure. Different superscripts (a, b, c, d, e) in a row indicate significant differences between the groups with $p < 0.05$.

Table 2
Parameters of the vitamin A transport complex in the study population.[a]

	Control	CKD 2	CKD 3	CKD 4	CKD 5
RBP4 (μg/mL)	44.9^a (29.6–63.6)	52.9^a (22.9–56.7)	64.5^{ab} (14.9–160.9)	88.2^b (37.2–133.8)	74.6^b (39.5–167.4)
TTR (μmoL/L)	339.9^a (215.1–548.9)	223.9^b (30.8–327.3)	281.1^{ab} (45.7–665.5)	349.3^a (105.6–499.4)	193.1^b (87.5–573.7)
RBP4-TTR ratio	0.32^a (0.16–0.64)	0.63^b (0.41–2.87)	0.58^b (0.25–1.05)	0.64^b (0.40–1.44)	1.34^c (0.44–2.24)
RBP4-L (% peak height RBP4)	45.5 (0.0–200.0)	56.9 (13.3–128.6)	82.8 (4.5–220.0)	66.7 (11.5–466.7)	78.5 (16.7–163.6)
RBP4-LL (% peak height RBP4)	0.0^a (0.0–50.0)	7.6^{ab} (0.0–26.3)	19.2^b (0.0–175.0)	11.1^b (0.0–333.3)	10.5^b (0.0–500.0)

[a] All data are given as median and range. Abbreviations used: CKD 2–5, chronic kidney disease stage 2–5; RBP4, retinol-binding protein 4; RBP4-L, RBP4 truncated at the last C-terminal leucine; RBP4-LL, RBP4 truncated at both C-terminal leucines; TTR, transthyretin. Different superscripts (a, b, c, d) in a row indicate significant differences between the groups with $p < 0.05$.

was lowest in the control group in comparison to CKD 3, CKD 4 and CKD 5 ($p < 0.05$). There were no significant changes in the relative amount of RBP4-L, however, the parameter tended to be elevated with progression of CKD. The TTR serum concentration was subjected to fluctuations with higher TTR serum levels in controls and CKD 4 in comparison to CKD 2 and CKD 5 ($p < 0.01$ for both), with no differences in CKD 3 to any other group. The RBP4-TTR ratio showed lowest values in the control group ($p < 0.001$) and continuously rose with progression of CKD with highest values in CKD 5 ($p < 0.01$ in comparison to controls, CKD 3 and CKD 4 and $p < 0.05$ in comparison to CKD 2, Table 2).

Correlation of RBP4 and TTR serum concentration and relative amounts of RBP4 species with parameters of diabetes mellitus and lipid metabolism

Several authors suggested an association of RBP4 with the development of insulin resistance and type 2 diabetes mellitus as well as with parameters of lipid metabolism. Therefore, the correlation of RBP4 and TTR serum concentration, RBP4-TTR ratio as well as the relative amounts of truncated RBP4 species with fasting glucose, HbA1c, BMI, blood pressure, total cholesterol, HDL- and LDL-cholesterol, triacylglycerol concentrations and eGFR were analysed (Table 3). All parameters revealed a strong inverse correlation with eGFR ($p < 0.001$ for all). In contrast, none of the parameters showed any significant association with BMI, systolic or diastolic blood pressure. Additionally, neither RBP4 serum concentration nor any of the RBP4 species were correlated with the diabetes-related parameters fasting glucose and HbA1c or to the parameters of lipid metabolism total cholesterol, HDL- and LDL-cholesterol concentration. However, RBP4 serum concentration was correlated with triacylglycerol concentration. Interestingly, TTR serum concentration and to a more pronounced extend

Table 3
Significant Spearman correlation coefficients of RBP4 and TTR serum concentrations, RBP4-TTR ratio and the relative amounts of the RBP4 species RBP4-L and RBP4-LL with parameters of diabetes mellitus and lipid metabolism as well as eGFR.[a]

	RBP4	TTR	RBP4-TTR ratio	RBP4-L	RBP4-LL
FG	–	−0.250*	0.224*	–	–
HbA1c	–	–	0.268*	–	–
BMI	–	–	–	–	–
SBP	–	–	–	–	–
DBP	–	–	–	–	–
Total cholesterol	–	0.412***	−0.532***	–	–
HDL-cholesterol	–	0.281**	−0.344**	–	–
LDL-cholesterol	–	0.373***	−0.486***	–	–
Triacylglycerides	0.327**	–	–	–	–
eGFR	−0.457***	0.300**	−0.674***	−0.318**	−0.362**

[a] Abbreviations used: BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FG, fasting glucose; RBP4, retinol-binding protein 4; RBP4-L, RBP4 truncated at the last C-terminal leucine; RBP4-LL, RBP4 truncated at both C-terminal leucines; SBP, systolic blood pressure. Statistically significant Spearman–Rho correlation coefficients are indicated with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

RBP4-TTR ratio revealed an association with fasting glucose ($p < 0.05$ for both), total cholesterol ($p < 0.001$ for both), HDL-cholesterol ($p < 0.01$ for both) and LDL-cholesterol ($p < 0.001$ for both). The RBP4-TTR ratio, but not TTR serum concentration, was positively associated with fasting glucose concentration ($p < 0.05$).

Discussion

It is well established, that the concentration of RBP4 is elevated in serum of patients with chronic kidney disease [10–13], probably caused by a decreased ability of the kidneys to filter and degrade

RBP4 in proximal tubular cells [6,22,23]. In accordance with these observations we confirm that serum RBP4 levels increased in parallel with progression of CKD. Several authors suggested an association of RBP4 serum concentration with diabetes-related parameters [1,2,24]. However, in the present study we found no associations between RBP4 serum levels and diabetes-related parameters, supporting recent publications, which demonstrated an influence of kidney function rather than of diabetes mellitus on RBP4 serum concentration [3–5]. Moreover, correlation between RBP4 serum concentration and triacylglycerol but not cholesterol levels could be shown, which is in contrast to previous studies [25,26].

In the present study, we confirmed previous results concerning the augmented appearance of truncated RBP4 species in patients undergoing hemodialysis [15,16]. Additionally, we were able to demonstrate the occurrence as well as the relative amounts of the truncated RBP4 species RBP4-L and especially RBP4-LL are associated with kidney function and substantially change during progression of CKD.

Thereby, we were able to demonstrate differing characteristics in the occurrence of the truncated RBP4 species. Single truncated RBP4-L was detected in all subjects except one of the study population and no differences in the relative amounts of RBP4-L were obvious between the study groups. Therefore, the appearance of RBP4-L might be physiological, which confirms previous results of Nedelkov et al. [27]. In contrast, the appearance of the RBP4-LL seems to depend on kidney function, since it was characteristic for CKD patients with no differences in frequency between diabetic and non-diabetic subjects (data not shown). Additionally, the relative amounts of RBP4-LL gradually increased with progression of CKD, which may suppose importance of renal tissue in the metabolism of the RBP4-LL. The potential function of the kidneys in the formation of truncated RBP4-forms is underlined by a previous study from our group showing that liver dysfunction does not affect the formation of RBP4-L and RBP4-LL [28]. Furthermore, the absence of any correlation of the relative amounts of RBP4-L and RBP4-LL with diabetes-related parameters and/or parameters of lipid metabolism supports the assumption that diabetes or lipid parameters do not relevantly affect RBP4-L and RBP4-LL levels.

Potential mechanisms of truncation as well as the functional role of truncated RBP4 species are not fully understood. However, the appearance of RBP4 species with truncation not exceeding that of both C-terminal leucines might point to a specific protease.

Jaconi et al. proposed that the truncation of RBP4 is a physiological process, which might serve as signal for the elimination and degradation of the protein [15]. The finding of the present study concerning RBP4-L may support this hypothesis, because RBP4-L was found in comparable amounts in the study population. In addition, it has been demonstrated that truncated RBP4, isolated from urine of patients with acute renal failure, inhibits the function of polymorph nuclear leucocytes [29], indicating a potentially non-physiological nature and function of truncated RBP4.

A further finding of this study was a strong association of RBP4-TTR ratio with parameters of diabetes mellitus and lipid metabolism, namely fasting glucose concentration and HbA1c as well as with total cholesterol, HDL- and LDL-cholesterol concentrations and eGFR. The physiological RBP4-TTR ratio ranges about 0.4 [30]. A decreased RBP4-TTR ratio has been reported to indicate vitamin A deficiency [31], due to the fact that hepatic RBP4 but not TTR secretion is reduced during hypovitaminosis A [31]. However, little is known about the physiological meaning of an increased RBP4-TTR ratio. Usually, only one molecule of RBP4 is bound to one TTR tetramer, though there are up to two binding sites [6,9]. Mody et al. supposed that the molar excess of RBP4 is compensated by an increased number of RBP4 molecules per TTR tetramer, resulting in an increased stability and reduced clearance of RBP4 [32]. However, it is also possible that excess RBP4 contributes to the TTR-unbound RBP4 moiety in the circulation. In any case, the results of the correlation analysis indicate that RBP4-TTR ratio may be a more useful marker for diabetes mellitus and/or changes in lipid metabolism than RBP4 alone – especially in CKD patients. This seems rational, since the survival of CKD patients is partly influenced by diabetic and nutritional status [33], both being considered in the RBP4-TTR ratio with TTR as nutritional marker [9] and RBP4 as potentially diabetes-related factor [1].

In conclusion, the present study clearly demonstrates that the progression of CKD is not only associated with a gradual elevation of RBP4 serum levels but also with successive changes in the relative amounts of the truncated RBP4 species RBP4-L and RBP4-LL. The pathophysiological importance of truncated RBP4 remains to be elucidated. However, both, truncated RBP4 and related proteases may provide diagnostic and therapeutic potential. Additionally, RBP4-TTR ratio may be used to assess the metabolic situation of CKD patients.

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