Microalbuminuria is a major determinant of elevated plasma retinol-binding protein 4 in type 2 diabetic patients

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Plasma retinol-binding protein 4 (RBP4) may be a new adipokine linked to obesity-induced insulin resistance and type 2 diabetes. The impact of diabetic nephropathy on plasma RBP4 levels, however, is not known. We tested the hypothesis that microalbuminuria is associated with elevated plasma concentrations of RBP4 in type 2 diabetic subjects. Retinol, its binding protein and transthyretin (TTR) were measured in the plasma and urine of 62 type 2 diabetic subjects, 26 of whom had microalbuminuria. The results were compared to 35 healthy control subjects. Despite no differences in plasma retinol, concentrations of the RBP4 were significantly elevated in plasma of diabetic patients and significantly higher in those with microalbuminuria. The higher plasma levels of the binding protein in subjects with microalbuminuria were accompanied by both significantly elevated plasma TTR and increased urinary levels of RBP4. There were no correlations of plasma-binding protein levels and parameters of insulin resistance. Our study suggests that plasma RBP4 levels in type 2 diabetic patients are affected by incipient nephropathy. Therefore, further studies evaluating RBP4 as a regulator of systemic insulin resistance and type 2 diabetes will need to take renal function into consideration.

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Retinol-binding protein 4 (RBP4) was initially recognized as the primary carrier for vitamin A (retinol) in plasma.[1] The small protein (molecular weight ~21 kDa) is synthesized mainly by the hepatocytes and secreted into plasma bound to retinol and transthyretin (TTR) as ternary retinol–RBP4–TTR complex.[2] RBP4 expression is also present in extrahepatic organs including principal insulin-sensitive tissues such as skeletal muscle and white adipose tissue.[3] Recently, it was shown that RBP4 expression in adipose tissue is upregulated in a transgenic mouse model with an adipocyte-specific ablation of the insulin-sensitive GLUT4 resulting in elevated levels of plasma RBP4.[4] It was also reported that RBP4 is elevated in plasma of subjects with obesity, impaired glucose tolerance and type 2 diabetes mellitus as well as that elevated RBP4 is associated with parameters of the metabolic syndrome.[5–9] These results suggest that RBP4 may serve as a potential adipokine playing a role in obesity-induced insulin resistance and the development of type 2 diabetes mellitus.[10,11]

The central importance of RBP4 in the vitamin A metabolism is reflected in the homeostatic regulation of plasma retinol, which guarantees a constant and continuous supply of vitamin A to peripheral tissues.[2] Several patho-physiological conditions, however, might be the cause of substantial fluctuations of plasma RBP4. Reduced levels of plasma RBP4 reflect impaired synthesis and/or secretion of RBP4. This can arise from several hepatopathies, dietary deficiency of vitamin A or inflammation.[12–14] On the other hand, increased levels of plasma RBP4 have been described in patients with chronic renal failure, which is attributed to a reduced glomerular filtration and catabolism of RBP4 in the kidneys.[15,16] Moreover, the impaired catabolism of the RBP4 complex in the kidneys leads to the accumulation of a truncated variant of RBP4 in plasma of patients with chronic renal failure pointing to the central importance of kidney function in the regulation of plasma RBP4.[17]

Nephropathy is also a serious microvascular complication in type 2 diabetic patients.[18] The decrease in renal function at the beginning of diabetic nephropathy is characterized by slight glomerular dysfunction, which is closely linked to

elevated urinary albumin excretion in the range of microalbuminuria.[19] To our knowledge, the impact of nephropathy on the plasma vitamin A-transport complex and in particular on RBP4 in type 2 diabetic subjects has not yet been investigated. This aspect, however, is of specific relevance and needs to be addressed in the discussion on the importance of RBP4 as an adipokine and its role in the pathogenesis of insulin resistance and type 2 diabetes mellitus. We therefore propose that the presence of incipient nephropathy might contribute to elevated levels of RBP4 in type 2 diabetic subjects. For this, we examined the combined effect of both type 2 diabetes mellitus and the occurrence of microalbuminuria on variables of plasma RBP4 complex as well as their excretion in urine.

RESULTS

Anthropometric and clinical parameters

Anthropometric and clinical characteristics of type 2 diabetic patients and controls are shown in Table 1. As expected, body mass index (BMI), waist circumference, waist-to-hip ratio, systolic blood pressure and parameters of impaired glucose utilization, such as fasting glucose, insulin, and homeostasis model assessment of β-cell function and insulin resistance (HOMA-IR) score as well as concentrations of interleukin-6 (IL-6) were increased (all $P<0.001$) in subjects with type 2 diabetes mellitus. Diastolic blood pressure was significantly higher ($P<0.01$) in type 2 diabetic patients with microalbuminuria. Glycosylated hemoglobin (HbA1c) increased progressively from normoalbuminuric type 2 diabetic patients to patients with microalbuminuria ($P<0.001$). No differences in the plasma concentrations of cholesterol and low-density lipoprotein cholesterol were seen between diabetic groups or controls, whereas concentrations of high-density lipoprotein (HDL) cholesterol were lower ($P<0.001$) in the type 2 diabetic groups. Moreover, type 2 diabetic patients with microalbuminuria had significantly higher levels of plasma triglycerides ($P<0.01$) and nonesterified fatty acids (NEFA) ($P<0.05$). Finally, there were no significant differences in the concentration of plasma creatinine ($P=0.597$) and glomerular filtration rate (GFR) ($P=0.658$) estimated by the Modification of Diet in Renal Disease (MDRD) Study Group formula.

Biochemical variables of the RBP4–TTR–complex

There were no significant differences in concentration of plasma retinol between type 2 diabetic subjects and controls (Table 2). Concentrations of plasma RBP4, however, were elevated in normoalbuminuric type 2 diabetic patients ($P<0.05$) and were highest in diabetic subjects with microalbuminuria ($P<0.001$ microalbuminuric vs controls; $P<0.01$ microalbuminuric vs normoalbuminuric). The higher concentrations of plasma RBP4 were also reflected by a reduced molar ratio of plasma retinol to plasma RBP4 ($P<0.001$ microalbuminuric vs controls; $P<0.05$ microalbuminuric vs normoalbuminuric). Furthermore, plasma TTR was also elevated in microalbuminuric type 2 diabetic subjects as opposed to normoalbuminuric type 2 diabetic patients ($P<0.05$). We also tested whether the higher concentration of RBP4 in plasma of type 2 diabetic patients reflected a higher synthesis of RBP4 by adipose tissue or if it paralleled incipient diabetic nephropathy as measured by microalbuminuria. To clarify this, we investigated all subjects

Table 1 | Clinical and biochemical characteristics of type 2 diabetic patients and controls

		Type 2 diabetic subjects	
	Controls (n=35)	Normoalbuminuria (n=36)	Microalbuminuria (n=26)
Age (years)	49 (21–71)[a]	62 (37–76)[b]	63 (40–78)[b]
Sex (M/F)	14/21	18/18	12/14
BMI (kg/m^2)	26.2 (19.1–41.5)[a]	31.5 (22.3–49.3)[b]	32.0 (22.8–56.9)[b]
Waist circumference (cm)	89 (64–128)[a]	105 (82–147)[b]	102 (88–149)[b]
WHR	0.86 (0.65–1.21)[a]	0.93 (0.79–1.15)[b]	0.94 (0.80–1.07)[b]
SBP (mm Hg)	122 (88–160)[a]	138 (102–178)[b]	133 (109–184)[b]
DBP (mm Hg)	75 (61–100)[a]	80 (63–98)[a,b]	83 (64–108)[b]
Glucose (mmol/l)	4.91 (4.01–5.53)[a]	6.27 (2.66–9.87)[b]	6.43 (4.68–10.5)[b]
Insulin (mU/l)	5.43 (0.39–21.0)	11.4 (2.96–63.5)[b]	8.50 (0.89–27.3)[b]
HbA1c (%)	5.1 (4.2–5.7)[a]	5.8 (4.6–8.3)[b]	6.3 (5.5–10.3)[c]
IL-6 (pg/ml)	1.10 (0.59–3.78)[a]	2.68 (0.74–7.18)[b]	2.56 (1.05–25.2)[b]
HOMA-IR	1.08 (0.10–4.39)[a]	3.12 (0.74–21.3)[b]	2.70 (0.25–7.78)[b]
Cholesterol (mmol/l)	5.68 (3.46–6.66)[a,b]	5.19 (3.25–7.27)[a]	6.06 (2.44–8.23)[b]
LDL cholesterol (mmol/l)	3.45 (1.69–4.79)	3.22 (1.61–4.64)	3.70 (0.99–5.94)
HDL cholesterol (mmol/l)	1.53 (1.02–2.62)[a]	1.21 (0.59–2.29)[b]	1.23 (0.76–1.91)[b]
Triglycerides (mmol/l)	1.04 (0.43–2.64)[a]	1.31 (0.52–2.92)[b]	1.65 (0.80–4.24)
NEFA (mmol/l)	0.50 (0.10–1.44)[a]	0.60 (0.11–1.19)[a]	0.77 (0.35–1.70)[b]
Creatinine (μmol/l)	77.8 (63.2–103)	78.9 (51.0–103)	79.2 (54.2–125)
Creatinine clearance (ml/min per 1.73 m^2)	83.7 (58.5–101)	84.9 (62.0–112)	83.7 (42.6–141)
Urinary albumin (mg/l)	0 (0–19.9)[a]	0 (0–27.2)[b]	61.4 (32.1–449)[c]

BMI, body mass index; DBP, diastolic blood pressure; F, female; HbA1c, glycosylated hemoglobin; HDL, high-density lipoproteins; HOMA-IR, homeostasis model assessment of β-cell function and insulin resistance; LDL, low-density lipoproteins; M, male; NEFA, nonesterified fatty acids; SBP, systolic blood pressure; WHR, waist-to-hip ratio.
Data are expressed as median (range).
[a,b,c]Values with different superscripts in the same line are significantly different.

Table 2 | Biochemical variables of the RBP4–TTR complex in plasma and urine of type 2 diabetic patients and controls

		Type 2 diabetic subjects	
	Controls (n=35)	Normoalbuminuria (n=36)	Microalbuminuria (n=26)
Plasma-Retinol (μmol/l)	1.81 (1.15–2.68)	1.70 (0.40–2.40)	1.74 (1.09–3.02)
Plasma RBP4 (μmol/l)	1.59 (0.88–2.91)[a]	1.75 (0.90–3.01)[b]	2.22 (1.41–3.65)[c]
Retinol/RBP4 index[a]	1.17 (0.75–1.83)[a]	0.95 (0.25–1.64)[b]	0.81 (0.40 –1.15)[c]
Plasma TTR (μmol/l)	4.02 (1.68–9.06)	4.22 (1.55–7.83)[a]	5.20 (2.15–11.0)[b]
Plasma RBP4 (Da)	21 072 (21 017–21 088)[a]	21 064 (20 999–21 081)[a,b]	21 053 (20 970–21 079)[b]
Urinary RBP4 (nmol/l)	2.43 (0.94–6.43)[a]	2.56 (1.19–11.8)[a]	3.60 (1.65–25.3)[b]

RBP4, retinol-binding protein 4; TTR, transthyretin.
Data are expressed as median (range).
[a]The retinol/RBP4 index is the molar ratio of plasma retinol to plasma RBP4.
[b,c]Values with different superscripts in the same line are significantly different.

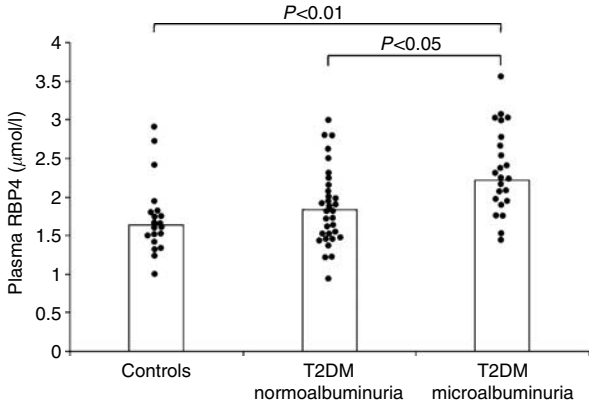

Figure 1 | RBP4 levels in plasma of controls ($n = 20$), subjects with type 2 diabetes mellitus (T2DM) and normoalbuminuria ($n = 32$), and subjects with T2DM and microalbuminuria ($n = 23$) adjusted for BMI (>25 kg/m^2). Plasma RBP4 was elevated in T2DM subjects with microalbuminuria compared to T2DM subjects with normoalbuminuria and controls (Mann–Whitney *U*-rank test). Scatter plot and bars show individual and median values, respectively.

Table 3 | Linear regression analysis using plasma RBP4 as the dependent variable and including age, gender, BMI, and presence of type 2 diabetes (A) or age, gender, BMI, and presence of microalbuminuria (B).

Parameter	Correlation	Standardized β	Correlation × standardized $\beta \times 100$ (%)	P-value
(A)				
Age	0.301	0.187	5.6	0.09
Gender	−0.14	−0.109	1.5	0.27
BMI	0.151	0.086	1.2	0.41
Type 2 diabetes	0.343	0.216	7.5	0.065
Total			15.8	
(B)				
Age	0.303	0.118	3.6	0.272
Gender	−0.154	−0.117	1.8	0.218
BMI	0.145	0.017	0.2	0.871
Microalbuminuria	0.443	0.373	16.5	0.002
Total			22.1	

BMI, body mass index; RBP4, retinol-binding protein 4.

with a BMI >25.0 with respect to their concentration of plasma RBP4 (Figure 1). Although there were indeed no significant differences for BMI and waist-to-hip ratio between the groups (data not shown), type 2 diabetic patients with microalbuminuria did have significantly elevated levels of plasma RBP4 as compared with normoalbuminuric type 2 diabetic subjects ($P = 0.032$) and controls ($P = 0.002$). However, under these conditions, the concentrations of plasma RBP4 did not differ between normoalbuminuric type 2 diabetic subjects and controls ($P = 0.211$).

In consideration of the effects of age, gender, BMI, microalbuminuria, and type 2 diabetes mellitus on parameters of the retinol-transport complex, we calculated a multivariate linear regression model aiming to predict levels of plasma RBP4. Using the presence of type 2 diabetes mellitus and presence of microalbuminuria within one model left microalbuminuria being significantly associated with plasma RBP4 ($P = 0.002$), whereas the presence of type 2 diabetes mellitus was no more associated (Table 3). Paradoxically, the higher concentrations of plasma RBP4 in microalbuminuric patients were accompanied by an increased excretion of RBP4 in urine ($P < 0.05$). We also assessed the presence of truncated molecular variants of plasma RBP4 by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis (Table 2). Mass differences of plasma RBP4 were lower (~ 19 Da) in type 2 diabetic patients with microalbuminuria as compared with controls ($P < 0.01$), but not with type 2 diabetic patients with normoalbuminuria.

Correlations among variables of the RRP4-transport complex and diabetic risk factors

The variables of the plasma RBP4-transport complex, retinol, RBP4 and TTR, were significantly and positively correlated with each other (data not shown). As depicted in Table 4, plasma retinol was negatively correlated with IL-6 and positively correlated with plasma triglycerides (both $P < 0.05$). Plasma RBP4 had a positive correlation with waist-to-hip ratio, fasting glucose, HbA1c, plasma triglycerides, NEFA (all $P < 0.001$), waist circumference ($P < 0.01$), systolic blood pressure, diastolic blood pressure, and urinary albumin (all $P < 0.05$), but not with BMI, fasting insulin, HOMA-IR, IL-6, and HDL cholesterol levels. The best

Table 4 | Significant Spearman rank correlations among variables of the retinol-RBP4-TTR-complex and diabetic risk factors.

	Retinol	RBP4	Retinol/RBP4 index	TTR	Urinary RBP4
BMI	—	—	−0.352**	—	—
Waist circumference	—	0.340	−4.449	—	—
WHR	—	0.329***	−0.283**	—	—
SBP	—	0.249*	−0.354**	—	—
DBP	—	0.216*	−0.314**	—	—
Glucose	—	0.330***	−0.493**	—	—
Insulin	—	—	−0.413**	—	—
HbA1c	—	0.420***	−0.615**	—	—
IL-6	−0.229*	—	−0.474**	—	—
HOMA-IR	—	—	−0.458**	—	—
HDL cholesterol	—	—	0.381***	—	—
Triglycerides	0.222*	0.453***	−0.345**	—	0.239*
NEFA	—	0.356***	−0.315**	—	—
Urinary albumin	—	0.197*	−0.288**	—	0.260*

BMI, body mass index; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP diastolic blood pressure; HbA1c, glycosylated hemoglobin; NEFA, non esterified fatty acids; LDL, low density lipoproteins; HDL, high density lipoproteins; HOMA-IR, Homeostasis model assessment of β-cell function and insulin resistance.
*$P<0.05$; **$P<0.01$; ***$P<0.001$.

variable correlated with diabetic risk factors was the calculated retinol/RBP4 index, which reflects the saturation of RBP4 with retinol. The retinol/RBP4 index was significantly negatively correlated to BMI, waist circumference, waist-to-hip ratio, systolic blood pressure, diastolic blood pressure, fasting glucose, insulin, HbA1c, IL-6, HOMA-IR, plasma triglycerides, NEFA, and urinary albumin (all $P<0.01$) and positively correlated with HDL cholesterol ($P<0.001$). Finally, urinary RBP4 excretion showed significantly positive correlation with urinary albumin excretion ($P<0.05$) and plasma triglycerides ($P<0.05$). Significant correlations between plasma TTR and any diabetic risk factor were not evident.

DISCUSSION

The levels of retinol, RBP4, and TTR in plasma are generally under homeostatic control.[2] However, it is also known that specific pathophysiological conditions such as vitamin A deficiency or inflammation are causing depressed levels of RBP4.[13] On the other hand, elevated levels of RBP4 can be observed in plasma of dialysis patients, even though they excrete RBP4 in urine.[15,16] Because in patients with type 2 diabetes mellitus both situations, an increased inflammatory status as well as an impairment of kidney function can exist,[19,20] we investigated in this study, whether the presence of microalbuminuria as an early marker of diabetic nephropathy is associated with changes in the concentrations of plasma RBP4 and also aimed to verify the hypothesis that high concentrations of plasma RBP4 are correlated with parameters of insulin resistance in type 2 diabetic patients. Our results showed that the higher concentrations of RBP4 in plasma of type 2 diabetic patients are not only linked to obesity, but might also be a consequence of incipient diabetic nephropathy as measured by microalbuminuria. Moreover, the linear regression model confirmed that the presence of microalbuminuria, but not type 2 diabetes mellitus alone was a significant contributor of elevated plasma RBP4. The findings also suggest that the role of RBP4 in systemic insulin action needs to be addressed through consideration of RBP4 as a primary regulator of plasma retinol homeostasis. Keeping this in mind, it is important to note that some organs other than adipose tissue are important sites of RBP4 synthesis and secretion.[21] In particular, there is compelling evidence showing that the kidneys play an important role in maintenance of whole-body retinol homeostasis,[22,23] which is regulated by glomerular filtration and subsequent reabsorption of RBP4 into the proximal tubular cells.[24,25] Thus, an impairment of the GFR results in an accumulation of RBP4 and other low-molecular weight proteins in the plasma.[12,26] In the study, although clearance measurements were not performed, the estimated GFR based on the MDRD Study Group formula was not different between the groups indicating that the presence of microalbuminuria was not related to changes in the GFR. Because the renal clearance of low-molecular weight proteins is close to the GFR,[27] it is therefore unlikely that changes in the GFR are responsible for the elevated RBP4 levels. Nevertheless, the lower molar ratios of retinol to RBP4 in plasma of type 2 diabetic subjects, which were lowest in patients with microalbuminuria, indicate the higher percentage of circulating apo-RBP4. This might be the result of a decreased renal uptake and catabolism of apo-RBP4 through a damaging effect of filtered protein on the proximal tubules.[28] Because apo-RBP4 has been considered as a physiological positive-feedback signal from peripheral tissues for the hepatic release of the RBP4 complex,[29] increased apo-RBP4 seems to be a plausible mechanism whereby RBP4 elevates in plasma of type 2 diabetic subjects with miocroalbuminuria. Further studies have to clarify if such a renal–hepatic pathway actually exists and if it is modulated in type 2 diabetic patients and whether the increased levels of apo-RBP4 stimulate the release of retinol–RBP4 complex from the liver or extrahepatic tissues.

In plasma, RBP4 is not only present as holo- (retinol-bound) and apo- (retinol-unbound) form, but also in different molecular variants, which can be detected by mass spectrometric analysis. Besides full-length RBP4, one parti-

cular RBP4 variant differing by the loss of one C-terminal leucine residue can be detected in normal plasma.[30,31] In plasma from patients with chronic renal failure, however, a truncated RBP4 variant with two C-terminal leucine losses is strongly evident,[30] a fact which prompted us to determine molecular masses of plasma RBP4 in type 2 diabetic patients using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. A significant difference in molecular masses of RBP4 between controls and type 2 diabetic patients with microalbuminuria was observed. However, the difference of about 19 Da is not indicative for the terminal loss of an amino-acid residue and suggests rather a rapid clearance of truncated RBP4 by the kidneys of type 2 diabetic patients. The cause of this mass difference is unknown and needs further investigation.

This study also considered the association between elevated RBP4, type 2 diabetes, and the inflammatory response. Because inflammation is thought to suppress the hepatic RBP4 mRNA synthesis,[32,33] plasma concentrations of both RBP4 and retinol decreased as a result of the inflammatory reaction.[13,14] Therefore, it is necessary to consider the inflammatory status of the subjects by measuring plasma IL-6, the main stimulator of acute-phase response.[34] Although in the study type 2 diabetic subjects had elevated concentrations of IL-6 in plasma, differences in plasma retinol were not evident suggesting that type 2 diabetes mellitus is a condition in which high concentrations of IL-6 are not associated with a decrease of plasma retinol. Interestingly, the higher IL-6 levels in subjects with type 2 diabetes mellitus were associated by increased levels of RBP4 in plasma, which is contradictory to the hypothesis of an inflammation-induced suppression of RBP4 synthesis by the liver.[32,33] It remains to be seen whether this paradoxical observation in the plasma retinol transport is attributed to so far unknown mechanisms in the obesity-induced inflammatory signaling pathways of type 2 diabetic subjects.

Although several studies have shown that RBP4 is indeed a secretion product of white adipose tissue,[3,4,21,35] further research is required to investigate whether RBP4 is causally involved in the pathogenesis of obesity and type 2 diabetes mellitus, and if so, determine whether RBP4-target therapies would be effective. One therapeutic agent that has been suggested is 4-(*N*-hydroxyphenyl) retinamide (fenretinide), a synthetic retinoid designed for cancer therapy. Fenretinide application in a mouse model has improved glucose tolerance and insulin sensitivity by lowering plasma RBP4, presumably through stimulation of RBP4 excretion in the kidney.[4] Our results, however, demonstrate that despite a higher excretion of RBP4 in urine, levels of RBP4 were elevated in plasma of type 2 diabetic subjects, a situation that was also reported by other investigators.[7] It remains therefore to be scrutinized whether fenretinide-induced urinary RBP4 excretion is feasible to reduce RBP4 in plasma of diabetic subjects also with regard to reported side effects of the retinol-transport system.[36] In addition, urinary excretion of RBP4 has long been recommended as a useful marker for the detection of minor changes in proximal tubular function long before the occurrence of elevation in other markers such as overt proteinuria or a rise in plasma creatinine.[37] RBP4 excretion is also associated with the excretion of other marker proteins of tubular proteinuria, such as albumin, β_2-microglobulin, or *N*-acetyl-β-D-glucosamidase.[38–40] Thereby, the severity of proteinuria is correlated with the decreased uptake of low-molecular weight proteins in the proximal tubule.[28] The positive correlation of urinary RBP4 with urinary albumin therefore corroborates that an increase in the filtered load of albumin may be responsible for increased urinary excretion of RBP4 by competition for the reabsorption sites in the proximal tubulus.[41] The results also implicate that incipient nephropathy in type 2 diabetic patients may be a renal disorder consisting of a tubulopathy as well as a glomerulopathy occurring simultaneously.

Although the sample size in this study was small, plasma RBP4 showed significant correlations with diabetic risk factors, such as waist circumference, systolic blood pressure, diastolic blood pressure, fasting plasma glucose, HbA1c, plasma triglycerides, and NEFA. We were, however, not able to find any significant correlation between plasma RBP4 and parameters of insulin resistance such as plasma insulin or HOMA-IR score. This is contrary to results of recently published studies.[4,8,9] Because the calculated molar ratio of retinol to RBP4 correlated negatively with almost all investigated diabetic risk factors, it is indicative that rather than holo-RBP4 itself, the actual amount of apo-RBP4 seems to play a crucial role in mediating RBP4-related effects on glucose metabolism. This suggestion, however, should be proven by further studies.

In conclusion, our results show that despite the presence of inflammation and increased renal excretion of RBP4, concentrations of RBP4 in plasma of obese type 2 diabetic patients were increased and were related to the presence of incipient nephropathy indicated by microalbuminuria. We therefore suggest that diabetic nephropathy is an additional factor that has to be considered in the discussion of RBP4 as an adipokine in obesity, insulin resistance, and type 2 diabetes mellitus.

MATERIALS AND METHODS

Subjects

A total of 62 adult patients with type 2 diabetes mellitus (American Diabetes Association criteria) were recruited from the MESY-BEPO cohort, which has been established by the Department of Endocrinology, Diabetes and Nutrition, Charité-University Medicine Berlin, Germany. The group consisted of 30 men and 32 women, aged between 37 and 78 years (median 63 years). They were divided according to urinary albumin excretion into two groups: 36 with normal urinary albumin excretion (<30 mg/l) and 26 with microalbuminuria (>30 mg/l). Diabetic patients were compared with 35 healthy subjects (14 men and 21 women), aged between 21 and 71 years (median age of 49 years). Blood was sampled after an overnight fast into EDTA tubes and centrifuged for plasma preparation. The subjects also provided a concurrent morning daytime specimen of urine. Aliquots of both, plasma and

urine were stored at −80°C until assayed. The study was approved by the Institutional Review Board, and an informed consent was obtained before the study from each subject.

Measurement of laboratory parameters

Anthropometry was performed as described previously.[20] Plasma samples were analyzed for glucose, insulin, cholesterol, low-density lipoprotein and HDL cholesterol, triglycerides and creatinine with a Cobas Mira Analyzer (Roche, Mannheim, Germany). The intra-assay coefficent of variation was: glucose 5.5%; insulin 6%; cholesterol 5.1%; HDL cholesterol 5.4%; and triglycerides 5.1%. Plasma NEFA was quantified using a colorimetric assay (NEFA, Wako, Neuss, Germany). Inter-assay coefficent of variation was 4.7%. IL-6 was determined by ELISA and HbA1c by high-performance liquid chromatography as described.[20] Urinary albumin was measured by immunological methods (ABX Diagnostics, Zurich, Switzerland) according to the manufacturer's instructions.

Analytical determination of plasma retinol, RBP4, and TTR

Concentrations of plasma retinol were measured using a modified gradient reversed-phase high-performance liquid chromatography-system (Waters, Eschborn, Germany) after organic extraction. For separation of the compounds, a reversed-phase C30 column (5 μm, 250 × 4.6 mm; YMC, Wilmington, USA) in line with a C18 pre-column (Luna, Phenomenex, Germany) was applied as described previously.[42] Retinol was quantified by measuring the absorption at 325 nm using an external retinol standard purchased from Sigma (Deisenhofen, Germany). The detection limit for retinol was 2.0 ng. Coefficient of variability over time using control plasma was $<4\%$ for retinol.

Concentrations of RBP4 in plasma and urine were quantitatively determined by ELISA as described previously.[43] Plasma TTR was measured by use of an ELISA technique adapted from the RBP4 procedure. In detail, wells of microtiter plates were coated by the addition of rabbit anti-human TTR IgG (DakoCytomation, Hamburg, Germany) diluted 1:2 000 in 50 μl of 50 mM carbonate buffer (pH 9.6), incubated for 1 h at 37°C, and stored at 4°C overnight. Plates were then washed four times with phosphate-buffered saline (PBS)-Tween (pH 7.4). For analysis, nonspecific binding was blocked by the addition of 0.5% bovine serum albumin diluted in PBS and incubated for 1 h at 37°C. After four additional washings, 50 μl of TTR standard (N Protein Standard/Standard SL OQIM 13, Dade Behring GmbH, Marburg, Germany), blank or plasma sample diluted with PBS (pH 7.4) containing 0.05% bovine serum albumin was placed in triplicate wells and incubated for 2 h at 37°C with constant shaking. After rinsing the wells four times with PBS-Tween (pH 7.4), 50 μl of peroxidase-conjugated sheep anti-human TTR IgG (Biotrend, Cologne, Germany), diluted 1:2 000 in PBS-Tween with 0.05% bovine serum albumin) was added to each well, and plates were further incubated for 1 h at 37°C. After four final washings, color was developed by the addition of *o*-phenylenediamine dihydrochloride solution (Sigma) and incubated for 20 minutes at 25°C. *o*-Phenylenediamine dihydrochloride solution (100 μl/well) consisted of 3.7 mM solution in 50 mM disodium phosphate–25 mM citric acid buffer (pH 5.2) containing 0.012% H_2O_2. The reaction was stopped through the addition of 1 M H_2SO_4 (50 μl/well), and absorbance was measured at 490 nm by use of a spectrophotometer (Microplate Reader, Bio-Rad, Munich, Germany). The inter-assay coefficient of variation was 8.1%.

Analysis of molecular variants of RBP4 by mass spectrometry

Molecular variants of RBP4 from plasma were assessed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry after initial isolation by sequential immunoprecipitation. Aliquots of 10 μl of plasma was mixed with 5 μl of anti-human RBP4 antibody (DakoCytomation) and incubated for 1 h at 25°C. The mixture was then spiked with 10 μl of Sephadex G-15 (1 mg/ml; Pharmacia, Uppsala, Sweden) to enhance the precipitation, vortexed, and incubated for 30 min at 25°C. After centrifugation (16 000 × *g*) for 20 min, the supernatant was removed. The pellet was then washed three times with 50 μl of PBS (pH 7.4), vortexed, and centrifuged (16 000 × *g*) for 10 min. The supernatants were discarded and the remaining pellet was resuspended in 10 μl of high-performance liquid chromatography-grade H_2O. For surface enhanced laser desorption/ionization time-of-flight mass spectrometry application, 1 μl of the dissolved pellet was deposited onto a spot of an aluminum ProteinChip array (Ciphergen Biosystems, Fremont, CA, USA). Finally, 1 μl of a saturated energy-absorbing molecule solution (EAM sinapinic acid; Ciphergen) dissolved in 50% acetonitrile and 0.5% trifluoroacetic acid was applied to the spot surface and the sample was allowed to dry. Mass analysis was performed in a ProteinChip Reader (PBS-II; Ciphergen) as described in detail elsewhere.[44]

Data analysis

Results were expressed as medians and ranges. HOMA-IR was calculated as fasting insulin (mU/l) × fasting glucose (mmol/l)/22.5.[45] The abbreviated MDRD Study Group formula was used to estimate the GFR based on age, sex, and serum creatinine (all patients were Caucasian): $\mathrm{MDRD} = 186.3 \times (\text{serum creatinine (mg/dl)})^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female})$ ml × min^{-1} per 1.73 m^2.[46] Statistical analysis was accomplished by use of nonparametric procedure (SPSS statistical package, version 12.0, SPSS Inc., Chicago, IL, USA). The Kruskal–Wallis test was used to test for significant differences in continuous variables between the groups. If there was a significant effect, Mann–Whitney *U*-rank test was performed to describe differences in proportions between case and control subjects. To identify independent determinants of plasma RBP4 levels, linear regression analysis was performed. Potential effects of diabetes or diabetic nephropathy were investigated after adjustment for age, gender, and BMI as potential confounders. Spearman rank correlation coefficients were used to test the association between anthropometric as well as laboratory parameters and variables of RBP4 transport complex. Values of $P<0.05$ were considered significant.

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