Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 58 (2009) 1386–1392
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Factors that influence retinol-binding protein 4–transthyretin interaction are not altered in overweight subjects and overweight subjects with type 2 diabetes mellitus

Simone K. Frey[a], Joachim Spranger[b,c], Andrea Henze[a], Andreas F.H. Pfeiffer[b,c], Florian J. Schweigert[a], Jens Raila[a,*]

[a]*Institute of Nutritional Science, University of Potsdam, D-14558 Nuthetal (Potsdam-Rehbrücke), Germany*
[b]*Department of Clinical Nutrition, German Institute of Human Nutrition Potsdam-Rehbrücke, D-14558 Nuthetal (Potsdam-Rehbrücke), Germany*
[c]*Department of Endocrinology, Diabetes and Nutrition, Charité-University Medicine Berlin, Campus Benjamin Franklin, D-12203 Berlin, Germany*

Received 12 January 2009; accepted 1 May 2009

Abstract

Retinol-binding protein 4 (RBP4) is an adipokine bound in plasma to transthyretin (TTR), which prevents its glomerular filtration and subsequent catabolism in the kidney. Alterations of this interaction have been suggested to be implicated in the elevation of RBP4 that are thought to contribute to the development of insulin resistance associated with obesity and type 2 diabetes mellitus (T2DM). However, the factors linking RBP4 to TTR in humans are not clear. Therefore, this study evaluated parameters influencing the RBP4-TTR interaction and their relation to obesity and T2DM. The RBP4 and TTR levels were quantified in plasma of 16 lean controls, 28 overweight controls, and 14 overweight T2DM patients by enzyme-linked immunosorbent assay. Transthyretin isoforms involved in RBP4 binding were determined by linear matrix-assisted laser desorption/ionization–time of flight–mass spectrometry after RBP4 coimmunoprecipitation. Holo-RBP4 (retinol-bound) and apo-RBP4 (retinol-free) were assessed by immunoblotting using nondenaturating polyacrylamide gel electrophoresis. Plasma levels of both RBP4 and TTR did not differ among the groups of lean controls, overweight controls, and overweight T2DM subjects. Using RBP4 immunoprecipitation, 4 mass signals were observed for TTR representing native, *S*-cysteinylated, *S*-cysteinglycinylated, and *S*-glutathionylated TTR. No differences in peak intensity of TTR isoforms were observed among the groups. Moreover, no differences in the ratio of holo- and apo-RBP4 were evident. The results suggest that circulating RBP4 and TTR were not affected by human obesity or T2DM, which might be attributed to the absence of alterations of TTR isoforms and the ratio of holo- and apo-RBP4 that might modify the TTR-RBP4 interaction.

1. Introduction

Transthyretin (TTR) is a homotetrameric protein that binds to and transports thyroxin and vitamin A (retinol) in the blood [1]. The retinol transport is accomplished by complex formation with retinol-binding protein 4 (RBP4) to prevent the glomerular filtration and thus excessive loss of vitamin A and RBP4 in urine [2]. Elevated levels of RBP4 observed in obese mouse models of insulin resistance and in insulin-resistant human subjects with obesity have suggested RBP4 to be involved in the pathogenesis of type 2 diabetes mellitus (T2DM) [3,4]. However, determining a role for RBP4 in human obesity and insulin resistance on the basis of the current literature is controversial, with publications reporting both increased and decreased levels of RBP4 under these conditions [5-10]. Potential explanations for this discrepancy are methodological considerations of RBP4 measurements [11] or the presence of additional factors affecting RBP4 in insulin-resistant states such as diabetic nephropathy or nonalcoholic fatty liver disease [12-16]. Recently, a study investigated TTR in leptin-deficient *ob/ob* mice and showed that changes in the RBP4-TTR interaction may also be implicated in the pathogenesis of insulin resistance and glucose intolerance by enhanced RBP4-to-TTR binding and thus decreased RBP4 clearance from the

Institutional approval: The study protocol was approved by the local ethics committee (Charité-University Medicine Berlin), and a written informed consent was obtained prior to the study from all participants.

* Corresponding author. Tel.: +49 33200 88 532; fax: +49 33200 88 573.
E-mail address: jens.raila@uni-potsdam.de (J. Raila).

0026-0495/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2009.05.003

circulation [17]. However, in humans, this issue has not been investigated yet.

It has also been suggested that structural modifications of TTR, which diagnostically can be identified as TTR isoforms, might affect the binding affinity to RBP4. Each TTR monomer has a single cysteine residue in position 10 that can exist in the native form with a free sulfide group or as a mixed disulfide with the amino acid cysteine (cys-TTR) as well as the peptides cysteinylglycine (cysglyc-TTR) and glutathione (glut-TTR) [18]. Although it is not known whether specific TTR isoforms preferentially bind to RBP4, the interaction between RBP4 and TTR is also influenced by the amount of holo- (retinol-bound) and apo-RBP4 (retinol-free) [19]. As holo-RBP4 is known to possess a higher binding affinity to TTR, it is supposed that an increase in holo-RBP4 might contribute to a decreased clearance of RBP4 from serum and thus account for elevated RBP4 levels in systemic insulin resistance [17]. The present study was therefore conducted to evaluate parameters implicated in RBP4-TTR binding in human obesity and T2DM. For this purpose, we investigated levels of TTR and TTR isoforms as well as levels of RBP4 and the ratio of holo- to apo-RBP4 in plasma of T2DM patients and compared them with those obtained from lean and overweight controls.

2. Subjects and methods

2.1. Subjects and sampling

A total of 14 adult patients with T2DM (American Diabetes Association criteria) were compared with 28 overweight and 16 lean control individuals. The diabetes group consisted of 4 men and 10 women. Type 2 diabetes mellitus was defined as fasting blood glucose greater than 126 mg/dL, 2-hour glucose greater than 200 mg/dL in a 75-g oral glucose tolerance test (OGTT), or use of insulin or oral hypoglycemic agents. In control subjects, diabetes was excluded by OGTT. In addition, these individuals did not report any chronic disease. After an overnight fast, blood was sampled into EDTA tubes and centrifuged for plasma preparation. Aliquots of plasma were stored at −80°C until assayed. The study protocol was approved by the local ethics committee, and a written informed consent was obtained before the study from all participants.

2.2. Measurement of laboratory parameters

Anthropometry was performed as previously described [20]. Plasma samples were analyzed for glucose, insulin, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, triglycerides, and creatinine concentrations with a Cobas Mira Analyzer (Roche, Mannheim, Germany). The intraassay coefficients of variation (CVs) were as follows: glucose, 5.5%; insulin, 6%; cholesterol, 5.1%; HDL cholesterol, 5.4%; and triglycerides, 5.1%. Plasma nonesterified fatty acids were quantified using a colorimetric assay (NEFA; Wako, Neuss, Germany). Interassay CV was 4.7%. Hemoglobin A_{1c} (HbA_{1c}) was determined by high-performance liquid chromatography as described [20]. Concentrations of RBP4 and TTR were measured by noncommercial enzyme-linked immunosorbent assay using polyclonal rabbit anti-human antibodies (Dako-Cytomation, Hamburg, Germany) as previously described [12]. Transthyretin and RBP4 standards used in the enzyme-linked immunosorbent assay were isolated from human serum (N Protein Standard/Standard SL OQIM 13; Dade Behring, Marburg, Germany), representing the physiologic (nontruncated) RBP4 form. Interassay CVs were 4.2% and 8.1% for RBP4 and TTR, respectively.

2.3. RBP4 immunoblot analysis

Holo-RBP4 and apo-RBP4 were determined in vertical slab polyacrylamide gel electrophoresis under nondenaturating conditions with subsequent RBP4 immunoblotting. Briefly, the resolving gel was prepared using 12% acrylamide/bisacrylamide mixed with 0.05% ammonium persulfate and 0.075% TEMED as cross-linker in 0.375 Tris/HCl (pH 8.8). The stacking gel (4% acrylamide/bisacrylamide, 0.05% ammonium persulfate, 0.1% TEMED) was prepared in 0.125 mol/L Tris/HCl (pH 6.8). Ten microliters of serum, diluted 1:20 in sample buffer (0.125 Tris/HCl, 2.74 mol/L glycerol, 0.1 mmol/L bromophenol blue [pH 6.8]), was applied to each slot. The RBP4 immunoblot procedure was performed as previously described [21]. Band intensity of RBP4 was read with an imager (Bio-Rad, Munich, Germany), and the relative amounts of apo- and holo-RBP4 were determined using the Bio-Rad Quantity One software.

2.4. Immunoprecipitation of the RBP4-TTR complex and matrix-assisted laser desorption/ionization–time of flight–mass spectrometry

For immunoprecipitation of TTR bound to RBP4, 10 μL of serum was incubated with 5 μL of polyclonal rabbit anti-human RBP4 (A0040, DakoCytomation) at 25°C for 18 hours. For control experiments, a polyclonal rabbit anti-human TTR (A0002, DakoCytomation) was used. 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 minutes at 25°C. After centrifugation (13 000 *g*) for 20 minutes, the supernatant was removed; and the protein-antibody complex was washed twice with phosphate-buffered saline (pH 7.4) and once with 5 mmol/L HEPES. The pellet was resuspended in 10 μL high-performance liquid chromatography–grade water. Matrix-assisted laser desorption/ionization–time of flight–mass spectrometry (MALDI-TOF-MS) was performed as previously described [18]. As the ionization efficiencies of native TTR, cys-TTR, cys-glyc-TTR, and glut-TTR are similar, the peaks in the mass spectra reflect the relative amounts of native TTR. Therefore, peak

Table 1
Anthropometric and metabolic characteristics in plasma of lean controls, overweight controls, and overweight T2DM patients

	Lean controls	Overweight controls	Overweight T2DM	*P* value[a]
n (M/F)	16 (4/12)	28 (10/18)	14 (4/10)	
Age (y)	50.5 ± 13.0	51.7 ± 8.49	61.9 ± 7.82	.027
BMI (kg/m^2)	21.8 ± 2.32	29.5 ± 3.81	30.9 ± 5.43	.001
WHR	0.82 ± 0.07	0.88 ± 0.07	0.93 ± 0.09	.005
SBP (mm Hg)	116 ± 15	124 ± 19	132 ± 12	.009
DBP (mm Hg)	74 ± 8	79 ± 9	78 ± 7	.028
Glucose (mg/dL)	85.7 ± 9.54	87.7 ± 5.40	114 ± 13.1	.001
Insulin (μU/L)	5.82 ± 4.94	9.39 ± 4.90	13.4 ± 8.92	.001
HbA_{1c} (%)	5.40 ± 0.48	5.39 ± 0.51	5.82 ± 0.30	.001
HOMA-IR	1.26 ± 1.14	2.04 ± 1.09	3.86 ± 2.67	.001
ln ($\Delta I_{30}/\Delta G_{30}$)[b]	4.11 ± 0.48	4.53 ± 0.60	4.25 ± 1.00	.149
Total cholesterol (mg/dL)	193 ± 5.40	220 ± 8.10	204 ± 15.4	.048
LDL cholesterol (mg/dL)	115 ± 23.1	144 ± 34.3	120 ± 50.1	.023
HDL cholesterol (mg/dL)	59.4 ± 14.2	50.5 ± 10.8	50.9 ± 11.5	.067
Triglycerides (mg/dL)	90.2 ± 29.2	125 ± 60.1	168 ± 63.7	.001
Plasma creatinine (mg/dL)	0.88 ± 0.12	0.88 ± 0.12	0.87 ± 0.13	.732
eGFR (mL/[min 1.73 m^2])	79.7 ± 8.32	80.0 ± 14.9	79.5 ± 14.8	.976

Data are expressed as mean ± SD. BMI indicates body mass index; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure.
[a] Kruskal-Wallis test.
[b] Estimated Δinsulin 30 to Δglucose 30 = insulin 30 − insulin 0/glucose 30 − glucose 0.

heights were determined in a "valley-to-valley" procedure and are expressed as percentage of native TTR.

2.5. Data analysis

Results were expressed as means ± SD. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin (in microunits per liter) × fasting glucose (in millimoles per liter)/22.5 [22]. The abbreviated Modification of Diet in Renal Disease Study Group formula was used to estimate the glomerular filtration rate (GFR) based on age, sex, and serum creatinine [23]. Statistical analysis was performed using SPSS version 14.0 (Munich, Germany). 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. Values of *P* less than .05 were considered significant.

Table 2
Parameters of the RBP4-TTR complex in plasma of lean controls, overweight controls, and overweight T2DM patients

	Lean controls	Overweight controls	Overweight T2DM	*P* value[a]
RBP4 (μg/mL)	40.7 ± 7.82	45.8 ± 25.8	39.3 ± 8.69	.76
TTR (μg/mL)	323 ± 159	367 ± 206	337 ± 111	.89
RBP4/TTR index	0.31 ± 0.12	0.33 ± 0.08	0.32 ± 0.13	.78
Holo-RBP4 (%)	83.0 ± 3.15	85.7 ± 2.17	85.4 ± 4.87	.91
Apo-RBP4 (%)	17.0 ± 5.42	12.8 ± 2.28	13.4 ± 3.87	.49
Cys-TTR (% of native TTR)	388 ± 96.4	533 ± 156	530 ± 173	.29
Cys-glyc-TTR (% of native TTR)	69.6 ± 4.61	48.2 ± 24.7	50.3 ± 35.9	.85
Glut-TTR (% of native TTR)	68.0 ± 4.61	96.0 ± 23.9	85.5 ± 34.5	.61

Data are expressed as mean ± SD. The RBP4/TTR index is the molar ratio of plasma RBP4 to plasma TTR.
[a] Kruskal-Wallis test.

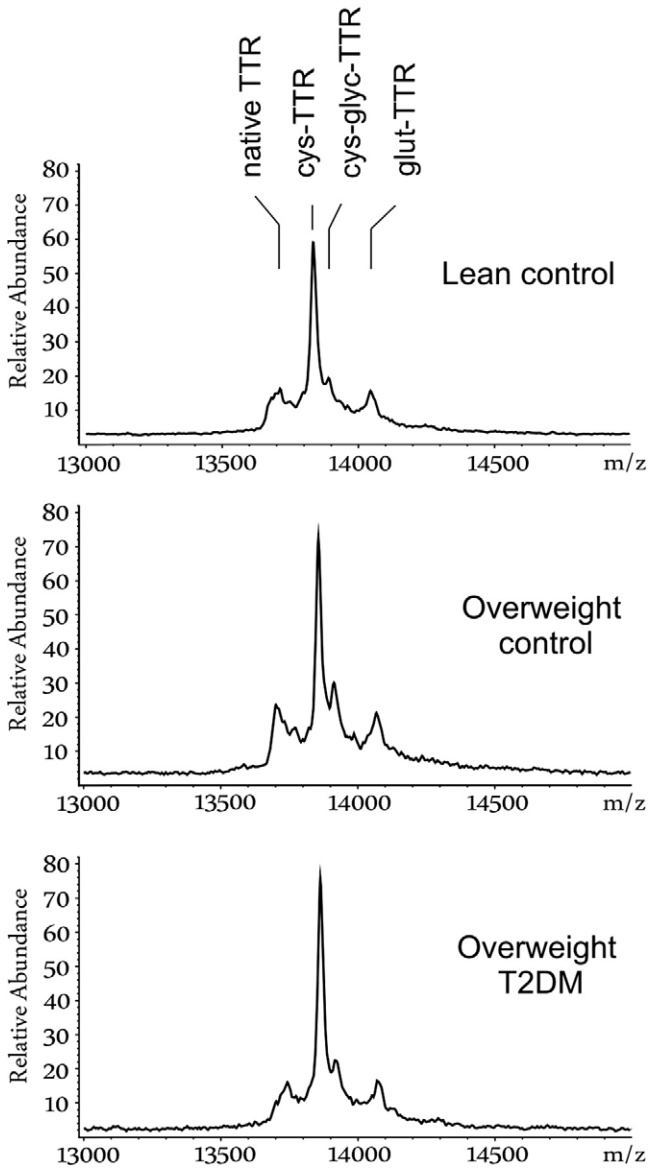

Fig. 1. Representative MALDI-TOF mass spectra display a similar profile of TTR isoforms isolated from plasma of lean controls, overweight controls, and overweight T2DM patients using RBP4 coimmunoprecipitation. Transthyretin isoforms consist of native TTR (molecular weight [MW] = 13 760 d), cysteinylated (cys-) TTR (MW = 13 870 d), cysteinglycinylated (cys-glyc-) TTR (MW = 13 924 d), and glutathionylated (glut-) TTR (MW = 14 060 d).

3. Results

3.1. Anthropometric and clinical parameters

Anthropometric and clinical characteristics of lean control subjects, overweight controls, and overweight subjects with T2DM are shown in Table 1. Overweight T2DM subjects were older ($P < .05$; Mann-Whitney test) compared with overweight but not lean controls. Body mass index and waist-to-hip ratio were higher in both overweight groups ($P < .01$). Compared with lean controls, parameters of impaired glucose metabolism, such as fasting plasma glucose, fasting insulin, and HOMA-IR, as well as concentrations of HbA_{1c} and triglycerides were increased (all Ps < .001) in overweight subjects with T2DM. Levels of insulin, HOMA-IR, cholesterol, and LDL cholesterol were also increased (all Ps < .01) in plasma of overweight controls compared with the lean controls. Between overweight controls and overweight T2DM subjects, differences were found in plasma glucose, HbA_{1c} (both Ps < .001), HOMA-IR ($P < .01$), and triglyceride levels ($P < .05$); but the ratio of the incremental response of insulin to glucose at 30 minutes of the OGTT ($\Delta I_{30}/\Delta G_{30}$) was not significantly different. With regard to kidney function, no significant differences in the concentration of plasma creatinine ($P = .73$, Kruskal-Wallis test) and GFR ($P = .98$) estimated by the Modification of Diet in Renal Disease Study Group formula were evident among the groups.

3.2. Levels of TTR and TTR isoforms

Quantitative plasma TTR concentrations did not differ between the groups (Table 2). After RBP4 immunoprecipitation, TTR spectra obtained by MALDI-TOF-MS

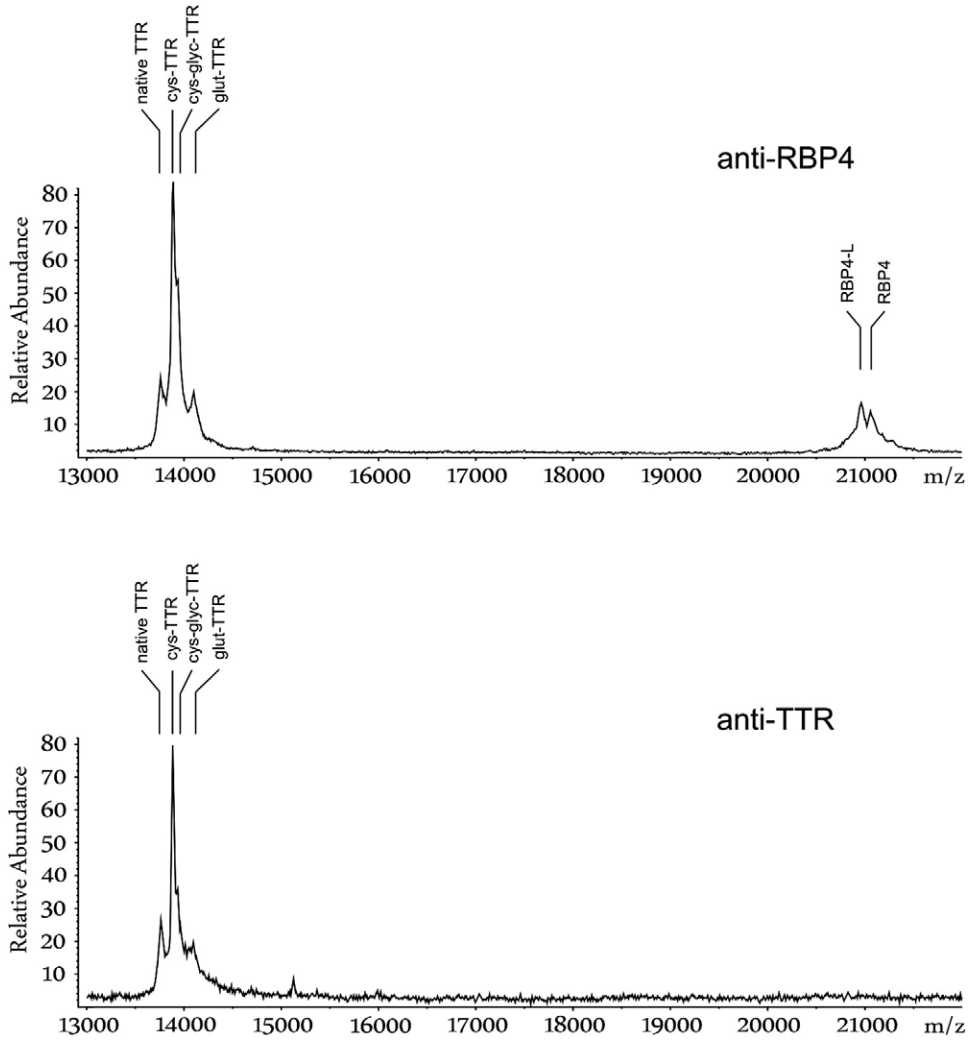

Fig. 2. Representative MALDI-TOF mass spectra of the RBP4-TTR complex from plasma of a lean control using coimmunoprecipitation with polyclonal RBP4 (anti-RBP4) or polyclonal TTR (anti-TTR) antibodies. Mass signals for TTR (~14 kd) are present in both spectra, whereas those for RBP4 (~21 kd) are only present using RBP4 immunoprecipitation. Transthyretin isoforms consist of native TTR, cysteinylated (cys-) TTR, cysteinglycinylated (cys-glyc-) TTR, and glutathionylated (glut-) TTR. Retinol-binding protein 4 isoforms are present as native nontruncated RBP4 and as RBP4-L, which is truncated at 1 C-terminal leucine molecule (Leu-183).

Table 3
Comparison of TTR isoforms in serum of healthy controls (n = 8) after immunoprecipitation using polyclonal TTR or RBP4 antibodies

TTR isoform	Anti-TTR IP	Anti-RBP4 IP	*P* value[a]
Cys-TTR (% of native TTR)	153 ± 25	162 ± 38	.85
Cys-glyc-TTR (% of native TTR)	51 ± 16	57 ± 13	.25
Glut-TTR (% of native TTR)	43 ± 13	50 ± 12	.19

Data are expressed as mean ± SD. IP indicates immunoprecipitation.
[a] Kruskal-Wallis test.

displayed 4 dominant peaks, which correspond to its native, unmodified form (mass = 13 760 d) and the following 3 Cys 10 adducts: cys-TTR (mass = 13 870 d), cys-glyc-TTR (mass = 13 924 d), and glut-TTR (mass = 14 060 d). In all groups, cys-TTR was the most abundant form, whereas peak intensity of cys-glyc-TTR, glut-TTR, and native TTR displayed lower intensity (Fig. 1). Moreover, no differences in the molecular masses of the individual signals were detected between lean/overweight controls and overweight type 2 diabetic subjects (Table 2). To determine whether RBP4 binding might affect structural modifications of TTR, we compared mass spectra from lean controls (n = 8) immunoprecipitated either with anti-RBP4 or with anti-TTR. When the RBP4-TTR complex was enriched through RBP4 antibodies, both RBP4 and TTR were detectable; but if a TTR antibody was used, only TTR but not RBP4 was found in the corresponding mass spectra, indicating that with this method the RBP4-unbound TTR fraction can be isolated (Fig. 2). However, despite this obvious difference in RBP4 binding, the relative amounts of TTR isoforms (cys-TTR, cys-glyc-TTR, and glut-TTR) were not different among the groups (Table 3).

3.3. Levels of RBP4 and relative amounts of apo- and holo-RBP4

The mean serum concentrations of RBP4 did not differ among the groups of lean controls, overweight controls, and overweight subjects with T2DM (Table 2). In addition, the ratio of holo- and apo-RBP4 did not vary among the groups.

4. Discussion

Several human and animal studies have investigated the influence of high circulating RBP4 levels in the pathogenesis of insulin resistance associated with T2DM and obesity [4-10]. Research has also focused on the metabolism of TTR, the physiologic binding protein of RBP4, in the blood [12,24,25]. Alterations of TTR-RBP4 interaction could play an important role in this context by stabilizing RBP4 at higher steady-state concentrations and thus impair the renal RBP4 clearance [17].

In the present study, we investigated TTR levels and, for the first time, TTR isoforms as well as the amount of holo- and apo-RBP4 as possible influencing factors of RBP4-TTR interaction in plasma of human subjects. The findings show that levels of both TTR and RBP4 in plasma were not altered in overweight subjects and overweight subjects with T2DM. With regard to RBP4, our results confirm previous investigations showing that circulating RBP4 is not related to insulin resistance in overweight human subjects [6,8,12]. Although the overweight T2DM subjects were older than overweight controls, plasma RBP4 did not differ between these groups, suggesting that there was no influence of age, which is in agreement with previous investigations [12,26-28]. In addition, levels of both RBP4 and estimated GFR (eGFR) were similar in the studied subgroups, confirming previous findings that, in the presence of normal renal function, circulating RBP4 is not affected by insulin resistance [12,13,27]. It was therefore suggested that additional factors might be considered in the discussion of RBP4 as a potential adipokine in the pathogenesis of insulin resistance. Hitherto, only nonalcoholic fatty liver disease and diabetic nephropathy have been reported as conditions that are related to elevated RBP4 levels in T2DM [12-16]. Recently, a study confirmed that the presence of impaired kidney function rather than T2DM determines the elevation of RBP4 [27].

Transthyretin has traditionally been seen not only as carrier for both RBP4 and thyroxin but also as a biomarker for nutritional status because its hepatic synthesis is mainly influenced by the adequacy of dietary protein and energy intake [29]. In our study, plasma TTR levels correspond with those of other investigations that assessed TTR as nutritional biomarker [30-32] and with previous results obtained from T2DM subjects [12]. Other studies have shown that TTR is elevated in subjects with impaired glucose tolerance or T2DM and that TTR is positively correlated with triglycerides and with LDL migration index [24,25]. Therefore, TTR was suggested as a marker of overnutrition [24]. However, our results do not support such a suggestion because of the absence of significant differences in TTR concentrations among the groups. In addition, normal renal function as attested by eGFR combined with the absence of elevated plasma TTR is consistent with the observation that TTR is significantly affected by renal function and therefore only elevated in patients with end-stage renal failure [30]. Moreover, we determined the molar ratio of plasma RBP4 to TTR and showed that the stoichiometry of these proteins was not altered in overweight controls and overweight T2DM subjects. In healthy states, 1 mol of RBP4 binds 1 mol of TTR tetramer; thus, the total molar concentration of plasma TTR exceeds that of RBP4 by 2.5 to 3.5 times [33]. This is consistent with our results and indicates that changes in RBP4/TTR stoichiometry are not affected by obesity and/or T2DM.

Transthyretin is important in maintaining circulatory levels of holo-RBP4 because it forms a large transport complex reducing the glomerular filtration of the relatively small holo-RBP4 molecule (21 kd) and therefore prevents its excessive loss in urine [2]. Moreover, TTR has a higher affinity to holo-RBP4 compared with apo-RBP4 because of

the retinol hydroxyl end group, which participates in the H-bond interactions with the TTR molecule [34]. Therefore, one can speculate that an elevation of holo-RBP4 may be stabilized by TTR binding that prevents RBP4 clearance. This might represent a potentially involved mechanism leading to an elevation of circulating RBP4. Here we show that neither the total amount of RBP4 nor the relative amount of holo-RBP4 was altered in both overweight subjects and overweight subjects with T2DM. The amount of holo- to apo-RBP4 is approximately 85% to approximately 15%, which is in agreement with those obtained in healthy subjects [16,35], whereas the ratio changes under certain conditions such as chronic kidney disease resulting from an increase of apo-RBP4 [36].

In the present study, we also assessed posttranslational modifications of the TTR molecule because structural modifications of TTR might also be involved in RBP4 retention [17]. The detection and characterization of such TTR modifications might be of importance not only for clinical diagnostics but also for our understanding of the pathogenesis of the disease [32,37,38]. The combination of immunoprecipitation and subsequent MALDI-TOF-MS allows the measurement of the molecular weight of intact TTR and the elucidation of the modified structures [32]. In the present study, we therefore used anti-RBP4 antibody to coimmunoprecipitate TTR bound to RBP4. In general, in our cohort, TTR was dominant in 4 variants, corresponding with previous studies by others and by our group [32,39,40]. All molecular variants including the unmodified native, cys-, cys-glyc-, and glut-TTR were detected in serum of controls and for the first time in serum of overweight T2DM subjects. Although there was no difference in peak-height ratio of TTR isoforms, the results clearly demonstrate that all coprecipitated TTR isoforms were involved in binding to RBP4. By contrast, no RBP4 could be detected if the complex was enriched through polyclonal TTR antibodies. With specific regard to TTR, there was no difference in the relative abundance of TTR isoforms irrespective of the antibody used for immunoprecipitation. These results show that the occurrence of TTR isoforms is not affected by the binding of RBP4 to the TTR molecule and indicate that the TTR isoforms do not differ in their affinity for RBP4 binding. We therefore suggest that structural modifications of TTR molecule might not be involved in altered RBP4 metabolism in overweight T2DM subjects.

In summary, we report findings showing that levels of RBP4 and TTR are not altered in overweight subjects and overweight subjects with T2DM. Moreover, factors stabilizing RBP4-TTR interaction and thus influencing RBP4 retention such as TTR isoforms and the ratio of holo- and apo-RBP4 were not affected in these groups. Further studies are necessary to define more fully the factors controlling each of these interactions and their relationships to the pathophysiologic function of the RBP4 transport system.

Acknowledgment

We are indebted to A Hurtienne, E Pilz, and L Häussler for expert technical assistance. JS was supported by a research group (Molecular Nutrition) of the *Bundesministerium für Bildung und Forschung* and a Heisenberg-Professorship of the *Deutsche Forschungsgemeinschaft*.

References

[1] Zanotti G, Berni R. Plasma retinol-binding protein: structure and interactions with retinol, retinoids, and transthyretin. Vitam Horm 2004;69:271-95.
[2] Goodman DS. Plasma retinol-binding protein. In: Sporn MB, Roberts AB, Goodman DS, editors. The retinoids. Orlando (Fla): Academic Press; 1984. p. 41-88.
[3] Yang Q, Graham TE, Mody N, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 2005;436:356-62.
[4] Graham TE, Yang Q, Bluher M, et al. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. N Engl J Med 2006;354:2552-63.
[5] Cho YM, Youn BS, Lee H, et al. Plasma retinol-binding protein–4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. Diabetes Care 2006;29:2457-61.
[6] von Eynatten M, Lepper PM, Liu D, et al. Retinol-binding protein 4 is associated with components of the metabolic syndrome, but not with insulin resistance, in men with type 2 diabetes or coronary artery disease. Diabetologia 2007;50:1930-7.
[7] Kowalska I, Straczkowski M, Adamska A, et al. Serum retinol binding protein 4 is related to insulin resistance and nonoxidative glucose metabolism in lean and obese women with normal glucose tolerance. J Clin Endocrinol Metab 2008;93:2786-9.
[8] Lewis JG, Shand BI, Frampton CM, et al. Plasma retinol-binding protein is not a marker of insulin resistance in overweight subjects: a three year longitudinal study. Clin Biochem 2008;41:1034-8.
[9] Gavi S, Qurashi S, Stuart LM, et al. Influence of age on the association of retinol-binding protein 4 with metabolic syndrome. Obesity (Silver Spring) 2008;16:893-5.
[10] Teranishi T, Ohara T, Maeda K, et al. Effects of pioglitazone and metformin on intracellular lipid content in liver and skeletal muscle of individuals with type 2 diabetes mellitus. Metabolism 2007;56: 1418-24.
[11] Graham TE, Wason CJ, Bluher M, et al. Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. Diabetologia 2007;50:814-23.
[12] Raila J, Henze A, Spranger J, et al. Microalbuminuria is a major determinant of elevated plasma retinol-binding protein 4 in type 2 diabetic patients. Kidney Int 2007;72:505-11.
[13] Ziegelmeier M, Bachmann A, Seeger J, et al. Serum levels of adipokine retinol-binding protein–4 in relation to renal function. Diabetes Care 2007;30:2588-92.
[14] Masaki T, Anan F, Tsubone T, et al. Retinol binding protein 4 concentrations are influenced by renal function in patients with type 2 diabetes mellitus. Metabolism 2008;57:1340-4.
[15] Seo JA, Kim NH, Park SY, et al. Serum retinol-binding protein 4 levels are elevated in non-alcoholic fatty liver disease. Clin Endocrinol (Oxf) 2008;68:555-60.
[16] Frey SK, Henze A, Nagl B, et al. Effect of renal replacement therapy on retinol-binding protein 4 isoforms. Clin Chim Acta 2009;401: 46-50.
[17] Mody N, Graham T, Tsuji Y, et al. Decreased clearance of serum retinol binding protein and elevated levels of transthyretin in insulin-resistant *ob*/*ob* mice. Am J Physiol Endocrinol Metab 2008;294: E785-93.

[18] Gericke B, Raila J, Deja M, et al. Alteration of transthyretin microheterogeneity in serum of multiple trauma patients. Biomarker Insights 2007;2:299-306.
[19] Peterson PA. Studies on the interaction between prealbumin, retinol-binding protein, and vitamin A. J Biol Chem 1971;246:44-9.
[20] Spranger J, Kroke A, Mohlig M, et al. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)–Potsdam Study. Diabetes 2003;52:812-7.
[21] Frey SK, Nagl B, Henze A, et al. Isoforms of retinol binding protein 4 (RBP4) are increased in chronic diseases of the kidney but not of the liver. Lipids Health Dis 2008;7:29.
[22] Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care 2004;27:1487-95.
[23] Kuan Y, Hossain M, Surman J, et al. GFR prediction using the MDRD and Cockcroft and Gault equations in patients with end-stage renal disease. Nephrol Dial Transplant 2005;20:2394-401.
[24] Yoshida A, Matsutani Y, Fukuchi Y, et al. Analysis of the factors contributing to serum retinol binding protein and transthyretin levels in Japanese adults. J Atheroscler Thromb 2006;13:209-15.
[25] Kloting N, Graham TE, Berndt J, et al. Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass. Cell Metab 2007; 6:79-87.
[26] Smith FR, Raz A, Goodman DS. Radioimmunoassay of human plasma retinol-binding protein. J Clin Invest 1970;49:1754-61.
[27] Henze A, Frey SK, Raila J, et al. Evidence that kidney function but not type 2 diabetes determines retinol-binding protein 4 serum levels. Diabetes 2008;57:3323-6.
[28] Erikstrup C, Mortensen OH, Nielsen AR, et al. RBP-to-retinol ratio, but not total RBP, is elevated in patients with type 2 diabetes. Diabetes Obes Metab 2009;11:204-12.
[29] Ingenbleek Y, Young V. Transthyretin (prealbumin) in health and disease: nutritional implications. Annu Rev Nutr 1994;14:495-533.
[30] Cano N, Di Costanzo-Dufetel J, Calaf R, et al. Prealbumin-retinol-binding-protein-retinol complex in hemodialysis patients. Am J Clin Nutr 1988;47:664-7.
[31] Ferard G, Gaudias J, Bourguignat A, et al. C-reactive protein to transthyretin ratio for the early diagnosis and follow-up of post-operative infection. Clin Chem Lab Med 2002;40:1334-8.
[32] Gericke B, Raila J, Sehouli J, et al. Microheterogeneity of transthyretin in serum and ascitic fluid of ovarian cancer patients. BMC Cancer 2005;5:133.
[33] Zago LB, Dupraz H, Sarchi MI, et al. The molar ratio of retinol-binding protein to transthyretin in the assessment of vitamin A status in adults. Proposal of a cut-off point. Clin Chem Lab Med 2002;40:1301-7.
[34] Raz A, Shiratori T, Goodman DS. Studies on the protein-protein and protein-ligand interactions involved in retinol transport in plasma. J Biol Chem 1970;245:1903-12.
[35] Sapin V, Alexandre MC, Chaib S, et al. Effect of vitamin A status at the end of term pregnancy on the saturation of retinol binding protein with retinol. Am J Clin Nutr 2000;71:537-43.
[36] Jaconi S, Saurat JH, Siegenthaler G. Analysis of normal and truncated holo- and apo-retinol-binding protein (RBP) in human serum: altered ratios in chronic renal failure. Eur J Endocrinol 1996;134:576-82.
[37] Sass JO, Nakanishi T, Sato T, et al. *S*-homocysteinylation of transthyretin is detected in plasma and serum of humans with different types of hyperhomocysteinemia. Biochem Biophys Res Commun 2003;310:242-6.
[38] Zhang Q, Kelly JW. Cys-10 mixed disulfide modifications exacerbate transthyretin familial variant amyloidogenicity: a likely explanation for variable clinical expression of amyloidosis and the lack of pathology in C10S/V30M transgenic mice? Biochemistry 2005;44:9079-85.
[39] Kiernan UA, Tubbs KA, Nedelkov D, et al. Comparative urine protein phenotyping using mass spectrometric immunoassay. J Proteome Res 2003;2:191-7.
[40] Schweigert FJ, Wirth K, Raila J. Characterization of the microheterogeneity of transthyretin in plasma and urine using SELDI-TOF-MS immunoassay. Proteome Sci 2004;2:5.