

CLINICAL STUDY

Retinol-binding protein 4 is associated with insulin resistance, but appears unsuited for metabolic screening in women with polycystic ovary syndrome

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Abstract

Objective: Adiposity, insulin resistance (IR), and hyperandrogenism are features of polycystic ovary syndrome (PCOS). Retinol-binding protein 4 (RBP4) secreted from adipose and liver tissues has been linked to IR. The impact of RBP4 on IR in PCOS and its usability to identify women with metabolic syndrome (MS) or impaired glucose tolerance (IGT) or diabetes were investigated.

Design: Plasma RBP4 was determined in 115 consecutive PCOS women. Associations with IR, body composition, and hyperandrogenemia were investigated by correlation and multiple linear regression analyses in 110 non-diabetics. Receiver operating characteristic curve analysis was used to evaluate RBP4 as a parameter for identifying MS and IGT or diabetes.

Results: RBP4 increased over tertiles of IR ($P=0.009$). RBP4 correlated with HOMA %S ($R=-0.286$, $P=0.002$), waist-to-hip ratio (WHR) ($R=0.233$, $P=0.034$), and dual energy X-ray absorptiometry (DEXA)-lean body mass ($R=0.282$, $P=0.016$) but not with body mass index (BMI), DEXA-total or trunk fat mass, hsCRP, free testosterone, DHEAS, androstenedione, and 17β -estradiol. Adjusted for age, BMI, smoking, and IGT, the association between RBP4 and HOMA %S remained significant ($P=0.032$). RBP4 explained 4.6% of the variation in HOMA %S. RBP4 was higher in MS and IGT or diabetes, but its ability to identify these women was low (area under the curve, AUC=0.631, $P=0.041$ or AUC=0.660, $P=0.016$).

Conclusions: In PCOS, RBP4 has a small independent impact on IR. It is not correlated with hyperandrogenemia, 17β -estradiol, other adrenal steroids, or with markers of adiposity in general. Furthermore, RBP4 does not appear suitable for screening MS or impaired glucose metabolism (IGT or diabetes).

European Journal of Endocrinology 158 517–523

Introduction

Polycystic ovary syndrome (PCOS) is one of the most frequent endocrine diseases in women (1, 2), characterized by hyperandrogenism, chronic anovulation, and infertility. Many PCOS women are overweight or obese and suffer from insulin resistance (IR) and other features of metabolic syndrome (MS), which increase the risk for type 2 diabetes mellitus (DM) and cardiovascular disease (3–5). There is evidence that adiposity and associated metabolic alterations such as IR play an important role in the development and maintenance of PCOS pathology at least in a substantial proportion of patients (3–5). On the other hand, hyperandrogenemia may enhance IR and metabolic dysregulation seen in these patients (6). This could lead to a kind of vicious cycle that may explain the finding

that women with PCOS are more insulin resistant than expected from age and body mass index (BMI) (7).

The pathophysiology that links adiposity, IR, and hyperandrogenism or chronic anovulation is only poorly understood. However, signals secreted from the adipose tissue are supposed to be involved (8). One of those factors is retinol-binding protein 4 (RBP4) that is secreted from the adipose tissue and the liver (9–12). In mice, overexpression of RBP4 has been shown to cause IR presumably by enhanced expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) and impairment of muscle insulin action (10). Therefore, RBP4 has been hypothesized to be a novel adipokine linking adiposity with systemic IR and potentially with adiposity-related disorders. In overweight women with PCOS compared with controls, enhanced expression of RBP4 in adipose tissue and

elevated serum RBP4 has been recently described (13). Interestingly, RBP4 expression and secretion from adipose tissue was enhanced by 17β -estradiol. In another cohort, no differences in serum RBP4 were observed between lean PCOS women and BMI-matched controls (14). In that study, RBP4 correlated with BMI, body fat mass, and waist circumference, but not with indices of IR (14). This contrasts to yet another report that describes an association with IR as assessed by a euglycemic-hyperinsulinemic clamp as well as with the waist-to-hip ratio (WHR) but not with BMI (15). As with the relationship of RBP4 to IR, parameters of adiposity and gonadal steroids are far from clear, we addressed the potential role of RBP4 in IR in PCOS women and tested whether RBP4 might be a useful tool for identifying insulin-resistant women with MS or for identifying women with impaired glucose metabolism (impaired glucose tolerance (IGT) or DM), as suggested (16).

Subjects and methods

Subjects

One hundred and fifteen consecutive PCOS women who were referred to our clinic between 2001 and 2006 because of hirsutism or oligo/amenorrhea were included after written informed consent was obtained. The cohort has been published previously in part (17–20). The diagnosis of PCOS was based on: a) the presence of chronic ovulatory dysfunction, i.e., oligomenorrhea (four or less cycles in the last 6 months) or amenorrhea (no cycles in the last 6 months); b) clinical signs of hyperandrogenism, i.e., hirsutism as defined by a Ferriman–Gallwey Score ≥ 8 (21); c) laboratory findings, i.e., hyperandrogenemia, defined as serum androgen levels (DHEAS, androstenedione, or total testosterone) above the upper limit of normal for the respective assay; and d) the exclusion of other disorders such as Cushing's syndrome, late-onset 21-hydroxylase deficiency, thyroid dysfunction, hyperprolactinemia, or androgen-secreting tumors. These diagnostic criteria for PCOS are consistent with the most commonly used diagnostic criteria for PCOS, often referred to as the NIH consensus criteria (22). Twelve patients were euthyroid under thyroid hormone replacement because of Hashimoto's thyroiditis. Six women had arterial hypertension and were normotensive under antihypertensive drugs. Five women suffered from asthma, allergic rhinitis, or hyperkinetic heart syndrome. One woman was under anti-epileptic medication. Ninety-one women were not taking any medication.

All women were studied within the first 10 days following menstruation in the case of mild oligomenorrhea or at random if they suffered severe oligo- or amenorrhea. Blood was sampled in the morning after an overnight fast, and the samples were stored at -20°C until analysis. An oral glucose tolerance test (OGTT) was performed in all women to define

glucose metabolism. Five patients were newly diagnosed as suffering from DM. If not stated otherwise the five women with DM according to the revised American Disease Association (ADA) criteria (23) were excluded from the analysis because of the endpoint IR. Among the remaining 110 women, 19 suffered from IGT. The clinical and endocrine features of these 110 women are given in Table 1. IR was quantified by calculating HOMA %S using the mean of three fasting glucose and insulin values (24) and the HOMA2 program kindly provided by Dr Levy.

Assessment of body composition

BMI was calculated as body weight (kg) divided by body height squared (m^2). Body fat and lean body masses were assessed in a subgroup of 73 women using whole-body scans by dual energy X-ray absorptiometry (DEXA; Lunar, Madison, WI, USA). Coefficient of variance was determined by repeated measurements and was 2.2% for total fat mass and 1% for lean body mass.

Assays

All biochemical and endocrine parameters were determined as described previously (18). Free testosterone was calculated from total testosterone and sex hormone-binding globulin as published (25) using a web-based calculator (<http://www.issam.ch/free-testo.htm>). The CAG repeat length polymorphism within the androgen receptor was determined in a subgroup of 61 women as described (26) and the mean CAG length was used for further calculations. For the measurement of RBP4, plasma samples were thawed for the first time and RBP4 was determined in

Table 1 Clinical and endocrine features of the non-diabetic polycystic ovary syndrome (PCOS) cohort ($n=110$).

| Characteristic | |
|--|--------------------|
| Age (years) | 28.67 \pm 0.51 |
| BMI (kg/m^2) | 31.60 \pm 0.75 |
| Fasting plasma glucose (mmol/l) | 4.61 \pm 0.06 |
| Fasting insulin (pmol/l) | 100.98 \pm 6.88 |
| HOMA %S | 76.78 \pm 4.33 |
| hsCRP (mg/l) | 4.07 \pm 0.60 |
| Calculated free testosterone (pmol/l) | 58.34 \pm 3.74 |
| Estradiol (pmol/l) | 252.92 \pm 28.26 |
| Progesterone (nmol/l) | 4.73 \pm 0.99 |
| LH (U/l) | 8.60 \pm 0.50 |
| LH/FSH | 1.82 \pm 0.18 |
| DHEAS (nmol/l) | 7.58 \pm 0.37 |
| Androstenedione (nmol/l) | 8.26 \pm 0.29 |
| SHBG (nmol/l) | 52.03 \pm 4.30 |
| 17-OH-progesterone (nmol/l) | 2.37 \pm 0.14 |
| DEXA-total fat mass (kg) | 34.15 \pm 1.44 |
| DEXA-lean body mass (kg) | 44.84 \pm 0.77 |
| Overweight/obese subjects (BMI > 25 kg/m^2) | 83 (75.5%) |
| Smoker | 6 (5.5%) |

Continuous variables are given as mean \pm s.e.m. and frequencies as n (%).

duplicate using a commercially available sandwich ELISA (lot no. 0604-304; Immundiagnostik, Bensheim, Germany), which is distributed in the United States by ALPCO Diagnostics (Salem, NH, USA). The intra- and interassay coefficients of variation were 3.9 and 5.1% respectively. According to the manufacturer's instruction, the polyclonal antibody used was generated against urinary RBP4, but detects both urinary and full-length RBP4. Full-length RBP4 serves as the standard in the ELISA and RBP4 can be measured both in plasma and serum samples.

Statistical analysis

Statistical analyses were performed with SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA). Values are reported as mean \pm s.e.m. Significance was considered at two-tailed $\alpha < 0.05$. Correlation analyses were performed using the Spearman correlation coefficient. The non-diabetic women were divided into tertiles of HOMA %S. RBP4 was ln transformed to achieve normal distribution according to the Shapiro–Wilk test. Differences in ln-RBP4 between tertiles were compared by ANOVA. Differences in ln-RBP4 between women with or without the MS; (according to the ATPIII definition (27)) and women with or without IGT or DM were compared by Student's *t*-test. The relation between RBP4 and further variables with respect to IR (HOMA %S) was addressed by multiple linear regression analysis. The quality of the model was derived from the R^2 value. The impact of RBP4 on the dependent variable was calculated as standardized $\beta \times$ correlation $\times 100$. Receiver operating characteristic (ROC) curve analysis was used to define the power of RBP4 and HOMA %S to identify PCOS women with MS, IGT, or DM. ROC curves were compared for significant differences using MedCalc Software (MedCalc, Maria-kerke, Belgium).

Results

The clinical and endocrine characteristics of the non-diabetic women are depicted in Table 1. RBP4 ranged from 8.8 to 44.2 mg/l with a mean value of 21.99 ± 0.637 mg/l ($n = 110$).

RBP4 is associated with IR in PCOS women

To examine the association between RBP4 and IR, the study cohort was divided into tertiles of HOMA %S. Tertile 1 comprised the most insulin-resistant women (HOMA %S ranging from 13.6 to 49.2%) and tertile 3 contained the most insulin-sensitive women (HOMA %S ranging from 86.6 to 232%). The concentrations of RBP4 significantly increased with increasing IR as depicted in Fig. 1 (ln-RBP4 values were significantly different between the three groups, $P = 0.009$, tertile 1:

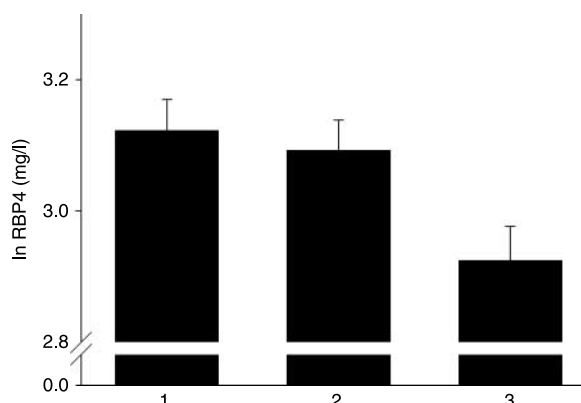


Figure 1 ln RBP4 levels in non-diabetic PCOS women categorized by tertiles of HOMA %S. Tertile 1 is the most insulin-resistant tertile. *P* value for difference is 0.009.

ln-RBP4 3.12 ± 0.05 mg/l, tertile 2: ln-RBP4 3.09 ± 0.05 mg/l, tertile 3: ln-RBP4 2.92 ± 0.05 mg/l). Furthermore, there was a significant correlation between RBP4 and HOMA %S ($R = -0.286$, $P = 0.002$, $n = 110$; Fig. 2). The association between RBP4 and HOMA %S was still significant after adjustment for age, BMI, smoking, and impaired glucose tolerance (IGT) using linear regression analysis (Table 2). In this model, RBP4 explained 4.6% of the variation in HOMA %S (Table 2). Further adjustment for parameters of hyperandrogenemia (free testosterone, DHEAS, and androstenedione; $\beta = -0.182$, $P = 0.018$, $n = 98$) and for drug use did not change the result.

The best model so far to explain the variability in HOMA %S in our cohort included age, smoking, IGT, BMI, WHR, free testosterone, the androgen receptor CAG repeat polymorphism length, and its multiplicative interaction with free testosterone (26). This model explained 58.1% of the variation in HOMA %S. The inclusion of RBP4 slightly improved that model which now explains 63.4% of the variation in HOMA %S. The impact of RBP4 on HOMA %S was calculated as 7.6% ($\beta = -0.242$, $P = 0.009$, $n = 61$), while age, smoking, WHR, and IGT had no significant impact.

In PCOS women, RBP4 is neither associated with parameters of adiposity in general nor with subclinical inflammation or hyperandrogenemia

The associations between RBP4 and determinants of IR in PCOS women, such as parameters of adiposity and hyperandrogenemia, were tested. Plasma RBP4 significantly correlated with WHR ($R = 0.233$, $P = 0.034$, $n = 83$) and DEXA-lean body mass ($R = 0.282$, $P = 0.016$, $n = 72$), but not with BMI ($P = 0.16$, $n = 109$), DEXA-total fat mass ($P = 0.54$, $n = 73$), or DEXA-trunk fat mass ($P = 0.50$, $n = 73$). The significance of the associations with WHR and DEXA-lean body mass were lost when the *P* values were corrected for the five endpoints used to

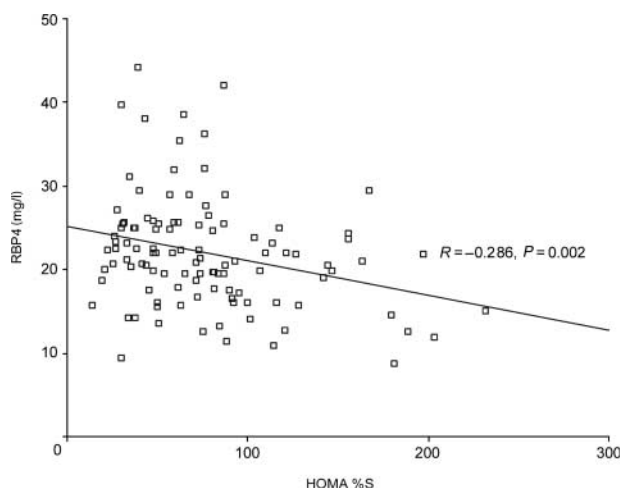


Figure 2 Correlation between RBP4 and HOMA %S in non-diabetic PCOS women.

describe body composition according to Bonferroni. As subclinical inflammation is associated with IR and adiposity, the association of plasma RBP4 with hsCRP was tested, which was negative ($P=0.287$, $n=74$). Furthermore, there was no correlation of RBP4 with free testosterone ($P=0.112$, $n=103$) and other androgens or sex steroids such as DHEAS ($P=0.211$, $n=106$), androstenedione ($P=0.581$, $n=108$), or oestradiol ($P=0.770$, $n=107$).

Is RBP4 useful in identifying PCOS women with the MS or impaired glucose metabolism?

RBP4 has been previously suggested as a potential marker to identify individuals with IR and a cardiovascular risk profile prior to the development of frank diabetes (16). We, therefore, tested whether RBP4 could be of value in identifying PCOS women suffering from the MS (defined according to Adult Treatment Panel III Criteria (27)) or from IGT or DM (23). Plasma RBP4 was elevated both in women with the MS (ln-RBP4 3.12 ± 0.04 mg/l, $n=29$ versus ln-RBP4 3.00 ± 0.04 , $n=70$, P value for difference: 0.027) and in women with IGT or DM (ln-RBP4 3.18 ± 0.05 mg/l, $n=24$ versus ln-RBP4 3.02 ± 0.03 , $n=91$, P value for difference: 0.018) as depicted in Fig. 3. ROC curve analysis was used to analyze the suitability of plasma RBP4 to identify PCOS

women with MS or with IGT or DM. The ROC curves for both endpoints (MS and IGT or DM) were significantly different from the bisecting line (MS: area under the curve (AUC) 0.631, $P=0.041$; IGT or DM: AUC 0.660, $P=0.016$). In our cohort, we have recently described that HOMA %S was the best-suited parameter to identify women with IGT or DM (28). We therefore compared RBP4 with HOMA %S. Concerning the endpoint MS, the AUC of the ROC curve for HOMA %S was significantly greater than the one for RBP4 (AUC HOMA %S 0.87, $P<0.001$ for the difference to the bisecting line, $P<0.001$ for the pairwise comparison with the ROC curve for RBP4). For the endpoint IGT or DM, the ROC curve for HOMA %S (AUC 0.74, $P<0.001$ for the difference to the bisecting line) was not significantly different from the ROC curve for RBP4 ($P=0.31$ for the pairwise comparison). We further compared the suitability of RBP4 and HOMA %S to predict IGT or DM by calculating the specificity at a given sensitivity of 95%. The specificity was 31% for RBP4 and 54% for HOMA %S at the 95% sensitivity cut point. Therefore, RBP4 compared with HOMA %S appears less apt to identify PCOS women with IGT or DM. This is in line with the assumption that in general an AUC between 0.6 and 0.7 indicates a poor test. Therefore, RBP4 measurements in PCOS women do not appear to be clinically useful to identify women who are at risk for cardiovascular disease, a pre-diabetic state or early DM.

Discussion

Adiposity, IR, and hyperandrogenism are key features in many women with PCOS (3–5). IR associates with metabolic disturbances that render PCOS women to an increased risk for type 2 diabetes and cardiovascular disease (29, 30). However, the molecular mechanisms involved in this complex interplay between adiposity, IR, hyperandrogenemia, and diabetic and cardiovascular risk factors are only incompletely understood.

RBP4, which is secreted from adipose and liver tissues (9–12), has been linked to IR in both rodent and human studies (10, 16, 31, 32). In our non-diabetic PCOS women who ranged from very insulin sensitive (HOMA %S 232.0%) to highly insulin resistant (HOMA %S 13.6%), RBP4 was significantly associated with HOMA %S, a well-introduced index of IR, which is derived from

Table 2 Multiple linear regression analysis with HOMA %S as the dependent variable ($R^2=0.478$). Impact of each significantly associated variable on HOMA %S as dedicated by the explanation of the variation in HOMA %S (calculated as standardized β coefficient \times correlation \times 100).

| | RBP4 (mg/l) | BMI (kg/m ²) | Age (years) | IGT (no/yes) | Smoking (no/yes) |
|--|----------------|--------------------------|----------------|----------------|------------------|
| Standardized β coefficient (P value) | -0.161 (0.032) | -0.547 (<0.001) | 0.274 (<0.001) | -0.205 (0.006) | -0.039 (0.59) |
| Correlation | -0.288 | -0.568 | 0.203 | -0.291 | -0.131 |
| Impact on HOMA %S (%) | 4.64 | 31.07 | 5.56 | 5.92 | |

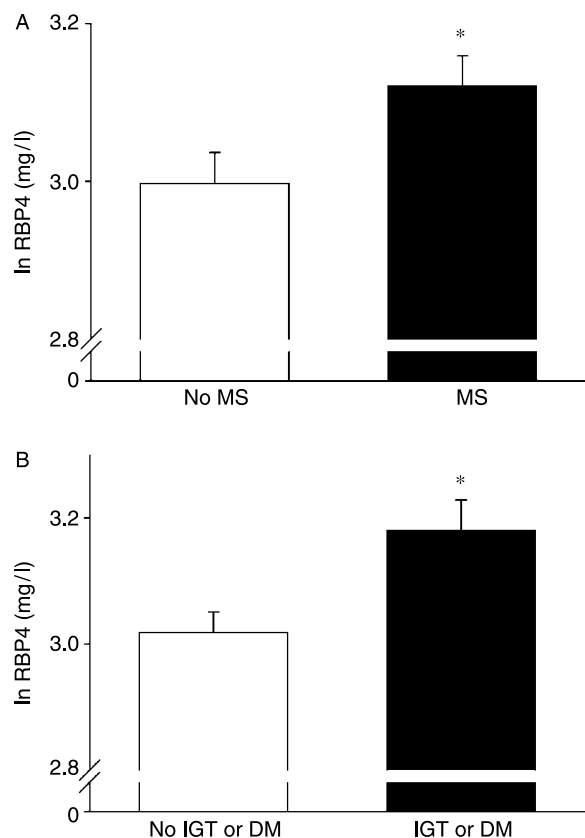


Figure 3 (A) ln RBP4 levels in PCOS women with or without metabolic syndrome, *P* value for difference is 0.027. (B) ln RBP4 levels in PCOS women with or without IGT or DM, *P* value for difference is 0.018.

parameters of the fasting state. The relationship between RBP4 and HOMA %S was still significant even after adjustment for BMI, age, smoking, and IGT. RBP4, however, explained only 4.6% of the variation in HOMA %S. In a multiple regression model, that included age, smoking, BMI, WHR, IGT, free testosterone, and the androgen receptor CAG repeat polymorphism length and its multiplicative interaction with free testosterone, RBP4 also had an independent impact on IR leading to further improvement of the model. Again, the impact of RBP4 on HOMA %S was relatively small explaining 7.6% of the variation in HOMA %S. Our finding is well in line with another study in PCOS women who showed an inverse relation between RBP4 and *M* value obtained during a euglycemic clamp study (15). HOMA %S correlates well with clamp data and has been introduced as a useful tool to assess IR in larger populations (24). However, to fully exploit its potential, the calculation of HOMA %S should be based on the mean of three fasting values for glucose and insulin and should be restricted to non-diabetic persons (24). These measures were recommended to minimize erroneous results caused by insulin pulsatility or advanced β -cell failure in DM (24). Thus, the relatively small impact of RBP4

on IR as described here, together with differences in the assessment of IR, may offer an explanation why the associations between RBP4 and IR in PCOS women as well as in other subjects reported so far are controversial (13–15, 31–34). Furthermore, cohort size, selection bias, or methodological differences in RBP4 measurements could contribute to obscure this relatively small impact on IR observed. Recently, a comparison of different methods and assays to measure RBP4 in blood has led to the suggestion that RBP4 should be preferentially determined by quantitative Western blotting in serum samples (35). Whether the measurement of RBP4 in serum by Western blotting is really superior and leads to different results and conclusions needs further evaluation. A recent paper by Yao-Borengasser *et al.* (34), in which RBP4 was measured in plasma and serum by the method used here, and by Western blotting, reported the same findings irrespective of the method of RBP4 measurement used. Furthermore, microalbuminuria has been described as a confounding factor that appears to increase RBP4-levels in diabetic patients (36). Since our analyses were restricted to non-diabetic patients significant distortion of the results by undiagnosed diabetic nephropathy appears unlikely.

In our PCOS patients with a range of BMI from underweight (18 kg/m²) to severe obesity (52 kg/m²), the WHR and DEXA-lean body mass were significantly associated with RBP4, whereas other parameters of adiposity or body composition like BMI, DEXA-total, or -trunk fat mass were not. The published data on the relation of RBP4 levels with various markers of obesity are inconsistent (9, 10, 16, 31, 32, 34, 37–40), which fits well to the result seen here showing that RBP4 is correlated with some but not all parameters of adiposity. In our study, the significance of the associations between RBP4 and WHR or DEXA-lean body mass were lost after correction for testing multiple endpoints. It therefore appears that there is only a weak (if any) significant link between circulating RBP4 and the degree of general adiposity in our cohort. Besides adipose tissue (11, 40), the liver is the second major source for RBP4 (12, 41) and data were published demonstrating an association of liver fat content with RBP4, rather than with other fat depots (32). Therefore, the possibility exists that the variable associations between RBP4 and markers of adiposity observed by us and others reflect the more or less stringent correlation between liver fat content and BMI or other markers of body composition in the respective cohorts.

RBP4 is secreted from hepatocytes into the blood stream bound to transthyretin (41, 42). Transthyretin is a negative acute phase protein that decreases in acute inflammation (43). Therefore, RBP4 may decrease in inflammatory states. On the other hand, enhanced inflammation in accumulating adipose tissue has been shown to correlate both with increased RBP4 expression from s.c. adipose tissue and with elevated plasma RBP4

(34). In our cohort of PCOS women, subclinical inflammation strongly associates with obesity and IR (18). We tested for an association between RBP4 and hsCRP as a central inflammatory marker. No correlation, however, could be delineated, which is line with a recent report from children (37). The data, however, reported from children are disputed (44). In PCOS women, we have no evidence for an association between RBP4 and inflammatory processes.

Very recently estradiol has been discussed as a putative regulator of RBP4, as its expression from human adipose tissue can be stimulated by 17 β -estradiol (13). Since relative hyperoestrogenemia as well as elevated androgen levels are key features of many PCOS patients, we investigated the relationship between sex steroids and RBP4 levels. We found, however, no associations between plasma RBP4 and systemic gonadal or adrenal steroids. This is in accordance with other studies in PCOS (14, 15) and with the finding that plasma RBP4 is not elevated in PCOS women *per se* (14), but contrasts to another report based on a small group of ten obese PCOS and control women, which described a very close relation between RBP4 and estradiol, testosterone, DHEAS, and androstenedione (13).

It has been reported that RBP4 is elevated even before the development of frank diabetes and appears to identify IR and associated cardiovascular risk factors in subjects with varied clinical presentations (16). Consistently, we found RBP4 levels higher in women suffering from MS or impaired glucose metabolism (IGT or DM) as has been reported previously from PCOS and other cohorts (14, 31, 45). ROC curve analysis, however, revealed that plasma RBP4 as determined here does not appear to be a clinically useful parameter to identify women suffering from either the MS, the IGT, or the DM. For both endpoints, the measurement of RBP4 was inferior to the calculation of HOMA %S.

Taken together, plasma RBP4 has a small, but independent, impact on IR in PCOS. RBP4 is neither correlated with hyperandrogenemia, 17 β -estradiol, other adrenal steroids, nor in general with markers of obesity. Despite RBP4 levels being increased in PCOS women with MS or impaired glucose metabolism (IGT or DM), RBP4 appears unsuited for the clinical screening of PCOS women for metabolic alterations such as MS or IGT and DM.

Acknowledgements

The authors thank B Faust and K Sprengel for their excellent technical assistance. There are no sources of external funding to declare. The study used departmental research funds allocated by the Charité and no conflict of interest exists.

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Received 21 December 2007

Accepted 3 January 2008