

Retinol-Binding Protein 4 and Insulin Resistance in Polycystic Ovary Syndrome

SAMANTHA K. HUTCHISON, MBBS^{1,2}
CHERYCE HARRISON, BBNSC^{1,3}
NIGEL STEPTO, PHD^{3,4}

CAROLINE MEYER, PHD²
HELENA J. TEEDE, PHD^{1,2}

OBJECTIVE — Polycystic ovary syndrome (PCOS) is an insulin-resistant state with insulin resistance being an established therapeutic target; however, measurement of insulin resistance remains challenging. We aimed to 1) determine serum retinol-binding protein 4 (RBP4) levels (purported to reflect insulin resistance) in women with PCOS and control subjects, 2) examine the relationship of RBP4 to conventional markers of insulin resistance, and 3) examine RBP4 changes with interventions modulating insulin resistance in overweight women with PCOS.

RESEARCH DESIGN AND METHODS — At baseline, 38 overweight women (BMI >27 kg/m²) with PCOS and 17 weight-matched control subjects were compared. Women with PCOS were then randomly assigned to 6 months of a higher-dose oral contraceptive pill (OCP) (35 µg ethinyl estradiol/2 mg cyproterone acetate) or metformin (1 g b.i.d.). Outcome measures were insulin resistance (total insulin area under the curve) on an oral glucose tolerance test, RBP4, and metabolic/inflammatory markers.

RESULTS — Overweight women with PCOS were more insulin resistant than control subjects, yet RBP4 levels were not different in women with PCOS versus those in control subjects (35.4 ± 4.3 vs. 28.9 ± 3.1 µg/ml, *P* = 0.36). RBP4 correlated with cholesterol and triglycerides but not with insulin resistance. Metformin improved insulin resistance by 35%, whereas the OCP worsened insulin resistance by 33%. However, RBP4 increased nonsignificantly in both groups (43.7 ± 6.3 vs. 42.6 ± 5.5 µg/ml, *P* = 0.92).

CONCLUSIONS — Overweight women with PCOS were more insulin resistant than control subjects, but this finding was not reflected by RBP4 levels. RBP4 correlated with lipid levels but not with insulin resistance markers. RBP4 levels did not change when insulin resistance was reduced by metformin or increased by the OCP. These data suggest that RBP4 is not a useful marker of insulin resistance in PCOS but may reflect other metabolic features of this condition.

Diabetes Care 31:1427–1432, 2008

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in reproductive-age women and affects 7% of this group and up to 30% of obese women (1). Reproductive abnormalities are underpinned by insulin resistance, which has a significant etiological role in PCOS (1). Women with PCOS have increased insulin resistance compared with that in control subjects (matched for BMI and body fat distribution) (1,2), as well as increased incidence

of metabolic syndrome (2), impaired glucose tolerance (IGT), and type 2 diabetes. However, insulin resistance is not included in the diagnostic criteria for PCOS. Challenges include inaccuracy of insulin assays, lack of clarity on optimal methods to assess insulin resistance, and ill-defined cutoff values to determine insulin resistance (2). A reproducible, accurate marker of insulin resistance that predicts outcomes and therapeutic responses would assist clinical management of PCOS.

Retinol-binding protein 4 (RBP4), an adipocyte product, is a carrier for vitamin A in blood. Although the majority of insulin-stimulated glucose uptake occurs in muscle, in insulin-resistant states, adipose tissue (not skeletal muscle) GLUT4 is downregulated (3). Recent convincing data in mice suggest a strong causal link between RBP4 and insulin resistance. Adipose-specific GLUT4 knockout mice exhibit insulin resistance with increased adipose RBP4 (3). Overexpression of RBP4 in mice induces insulin resistance, whereas decreases in RBP4 reduce insulin resistance. Furthermore, RBP4 levels can be normalized by insulin sensitizers and fenretinide, which reverse insulin resistance in obese rodents. Increased RBP4 impairs insulin signaling in muscle and increases gluconeogenesis in mouse liver, suggesting that RBP4 is involved in the pathogenesis of insulin resistance and is a marker of insulin resistance in mice.

In contrast, human data are equivocal, with high adipocyte and plasma RBP4 levels being reported inconsistently in insulin-resistant states, including obesity, IGT, type 2 diabetes, and PCOS (3–9). Relationships of RBP4 with features of the metabolic syndrome have also been inconsistently demonstrated (4,6,8). RBP4 has been shown to change with interventions that reduce insulin resistance, including weight loss (5,10), exercise (4), and insulin sensitizers, although results are variable (8,9). Methodological issues in the measurement of RBP4 may contribute to inconsistencies, with few studies using the recommended Western blot technique (11).

It is increasingly clear that PCOS is an insulin-resistant state and insulin resistance per se is an important therapeutic target. Assessment of insulin resistance is likely to guide treatment with options including the oral contraceptive pill (OCP) (which can increase insulin resistance) or metformin (which reduces insulin resistance) (2,12). However, significant challenges remain in the measurement of insulin resistance (2). Although RBP4 shows promise as a marker of insulin resistance in mice, its role in humans remains unclear. We aimed to clarify the role of serum RBP4 (using the Western blot technique) in PCOS by comparing

From the ¹Jean Hailes Foundation Research Group, Monash University Institute of Health Services Research, Melbourne, Australia; the ²Diabetes Unit, Southern Health, Melbourne, Australia; the ³Department of Physiology, Monash University, Melbourne, Australia; and the ⁴School of Human Movement, Recreation and Performance, Victoria University, Melbourne, Australia.

Corresponding author: Professor Helena Teede, helena.teede@med.monash.edu.au.

Received 9 December 2007 and accepted 1 April 2008.

Published ahead of print at <http://care.diabetesjournals.org> on 4 April 2008. DOI: 10.2337/dc07-2265.

Clinical trial reg. no. ISRCTN51367236, clinicaltrials.gov.

© 2008 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

RBP4 levels in overweight women with and without PCOS. We also examined the relationship between serum RBP4 levels and conventional insulin resistance markers, other metabolic factors, and adiposity in women with PCOS. Finally, we examined the effect of therapeutic interventions that both increase and decrease insulin resistance in women with PCOS on serum RBP4 levels.

RESEARCH DESIGN AND METHODS

This study comprises a subset of subjects from a larger pharmacologic intervention study (12). The subset reported here includes 38 women with PCOS who were sequentially recruited and randomly assigned to a treatment group and who completed the study intervention. Overweight women (BMI >27 kg/m²) with PCOS (*n* = 38) and overweight control subjects (*n* = 17) were recruited using community advertisements. A diagnosis of PCOS was based on perimenarchal onset of irregular cycles (<21 days or >35 days) and clinical hyperandrogenism (hirsutism or acne) or biochemical hyperandrogenism (elevation of at least one circulating ovarian androgen level) (1990 National Institutes of Health criteria). Secondary causes of amenorrhea and hyperandrogenism were excluded with clinical screening and early follicular 17-hydroxyprogesterone levels. Type 2 diabetes was excluded by an oral glucose tolerance test (OGTT) (World Health Organization criteria). Results of pregnancy tests were negative before enrollment. The Southern Health Research Advisory and Ethics Committee approved the study, and all participants gave written informed consent.

At screening (3 months before baseline), standard diet and lifestyle advice was delivered (National Heart Foundation of Australia recommendations), and medications affecting insulin resistance, including the OCP, were ceased. At baseline, women with PCOS were randomly assigned, on the basis of computer-generated random numbers, to either 1 g b.i.d. metformin (dose titrated up over 4 weeks starting at 500 mg b.i.d.) or a higher-dose OCP (35 µg ethinyl estradiol/2 mg cyproterone acetate) in an open-label study. The higher-dose OCP is a commonly prescribed OCP for women with PCOS in Australia and Europe. Participants were reviewed by the same investigator at screening, at baseline, and at 3 and 6 months after intervention. Data collection was completed

by the research nurse, who blinded to treatment allocation.

Clinical and biochemical measurements

Subjects were weighed while lightly clothed without shoes, BMI was calculated (weight in kilograms divided by height in meters squared), and waist and hip circumferences were measured at the umbilicus and greater trochanter. The waist-to-hip ratio was calculated as the ratio of waist circumference to hip circumference. Fasting blood samples were taken for endocrine and metabolic variables, and an OGTT was performed at randomization and at 6 months in the intervention groups.

Venous blood samples were collected after an overnight fast for assessment of glucose, insulin, testosterone, sex hormone-binding globulin (SHBG), total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, high-sensitivity C-reactive protein (hsCRP) (12), and RBP4. The free androgen index (FAI) was calculated as FAI = (testosterone/SHBG) × 100. A 120-min 75-g OGTT was performed, and blood samples were taken for assessment of glucose and insulin at 0, 60, 120, and 180 min. Total insulin area under the curve (AUC insulin) during the OGTT was calculated geometrically using the trapezoidal rule. Homeostasis model assessment (HOMA) was used as a surrogate measure of insulin sensitivity and was calculated as fasting serum insulin (milliunits per liter) × (fasting plasma glucose [millimoles per liter]/22.5) as described previously (12). Because of erratic menstrual cycles, data were not collected at a specific cycle stage in women with PCOS. Control data were collected during the follicular phase.

RBP4

Quantitative Western blotting was performed based on the methods described by Graham et al. (4,11). In brief, the full-length recombinant RBP4 protein concentration (Cayman Chemical, Ann Arbor, MI) was determined using the Bradford method (Bio-Rad, Hercules, CA). Standard solutions of 25, 50, 100, and 150 µg/ml RBP4 were prepared in a standard buffer containing 0.1% BSA and 1% Nonidet P-40. Human sera and standards were then diluted 1:10 into a 1× lithium dodecyl sulfate-PAGE sample buffer and heated for 5 min at 95°C. In addition to molecular weight markers, 15 µl of diluted standards and samples were

loaded on 16% Tris-glycine precast SDS-PAGE cassette gels (PAGEgel, San Diego, CA) and transferred to nitrocellulose membranes for immunoblotting. Nitrocellulose membranes were then blocked in solution containing 5% nonfat milk in PBS and Tween 20. Blots were probed overnight with primary antibody (anti-RBP4; Sapphire Bioscience, Redfern, NSW, Australia) diluted 1:500 at 4°C. After washing with PBS-Tween 20, blots were rocked for 1 h in secondary antibody (polyclonal goat anti-rabbit immunoglobulin horseradish peroxidase; DAKO Cytomation, Kingsgrove, NSW, Australia) diluted 1:1,000 at room temperature. Bands were detected by enzymatic chemiluminescence (Millipore, Billerica, MA) and quantified using a luminescence imaging program (Multigauge, FujiFilm, Tokyo, Japan).

Assays

Hormone, lipid, and inflammatory assays were completed as described previously (12).

Statistics

All data are means ± SEM and were log-transformed if not normally distributed. Results are presented for 55 subjects, 38 with PCOS who were treated with metformin (*n* = 19) or the OCP (*n* = 19) and 17 control subjects, except for the data regarding hsCRP (*n* = 43), for which 12 women with levels >10 mg/l, potentially attributable to other inflammatory processes, were excluded. Two-tailed statistical analysis was performed using SPSS for Windows 14.0 software (SPSS, Chicago, IL) with statistical significance set at an α level of *P* ≤ 0.05. Baseline data were assessed using one-way ANOVA with PCOS status as a between-subject factor. PCOS group was assessed using one-way ANOVA with intervention as a between-subject factor and comparisons between time points were assessed using repeated-measures ANOVA with intervention as a between-subject factor. Relationships between variables were examined using bivariate (Pearson) correlations. Change in variable was defined as the ratio of pretreatment value and posttreatment value.

RESULTS— All 17 control subjects and 38 women with PCOS were screened and then completed the 3 month run-in.

Table 2—Characteristics at baseline and study end: metformin and OCP group

Characteristic	Metformin		OCP		P value for change over study: metformin vs. OCP
	Before	After	Before	After	
<i>n</i>	19	19	19	19	
BMI (kg/m ²)	38.4 ± 1.6	37.7 ± 1.6	35.3 ± 1.8	35.3 ± 1.8	0.26
Weight (kg)	105.2 ± 4.7	103.4 ± 4.6	94.1 ± 5.0	94.2 ± 5.0	0.24
Waist circumference (cm)	112.6 ± 3.5	113.7 ± 4.0	105.1 ± 3.8	107.2 ± 3.5	0.52
Waist-to-hip ratio	0.9 ± 0	0.9 ± 0	0.9 ± 0	0.9 ± 0	0.65
hsCRP (mg/l)	4.2 ± 0.68	3.8 ± 0.73	4.6 ± 0.93	7.4 ± 2.0	0.03
Testosterone (nmol/l)	2.4 ± 0.1	2.2 ± 0.3	2.1 ± 0.2	1.7 ± 0.1	0.87
SHBG (nmol/l)	32.4 ± 4.4	43.2 ± 9.6	31.7 ± 2.8	133.7 ± 17*†	<0.01
FAI	9.9 ± 1.5	10.7 ± 2.7	8.5 ± 1.7	1.8 ± 0.3*†	<0.01
Cholesterol (mmol/l)	5.3 ± 0.3	5.1 ± 0.3	5.1 ± 0.2	4.9 ± 0.2	0.81
HDL cholesterol (mmol/l)	1.2 ± 0.1	1.1 ± 0.1*	1.4 ± 0.1	1.4 ± 0.1†	0.01
LDL cholesterol (mmol/l)	3.4 ± 0.3	3.2 ± 0.3	3.2 ± 0.2	2.7 ± 0.2*	0.15
Triglycerides (mmol/l)	1.6 ± 0.1	1.6 ± 0.2	1.2 ± 0.1	1.7 ± 0.2*	<0.01
Fasting glucose (mmol/l)	4.7 ± 0.2	4.6 ± 0.2	4.4 ± 0.1	4.3 ± 0.1	0.74
Fasting insulin (mU/l)	21.5 ± 4.2	17.8 ± 5	21 ± 4.4	20.8 ± 2.9	0.1
AUC insulin (mU · l ⁻¹ · 120 min ⁻¹)	12,333.9 ± 2,046.4	8,054.7 ± 1,090.6*	9,339.8 ± 1,531.5	12,413.5 ± 1,616.7*†	<0.01
HOMA	4.7 ± 1.1	4.2 ± 1.7	4.2 ± 0.9	4 ± 0.6	0.1
RBP4 (µg/l)	35.9 ± 7.4	43.7 ± 6.3	35.7 ± 5.3	42.6 ± 5.5	0.92

Data are means ± SEM. Baseline data were assessed using one-way ANOVA with intervention as the between-subject factors and intervention data were assessed using repeated-measures ANOVA with time as the within-subject factor and intervention as the between-subject factor. * $P < 0.05$ for within-group change over study intervention. † $P < 0.05$ for difference between OCP and metformin group at study beginning or end.

Control subjects versus women with PCOS at baseline

Baseline clinical, anthropometric, and endocrine characteristics are listed in Table 1. The control and PCOS groups were similar in age, BMI, and waist-to-hip ratio. Testosterone, FAI, HDL cholesterol, and triglycerides were higher in the PCOS group (Table 1). Two markers of insulin resistance, HOMA and fasting insulin, were higher in the PCOS group (Table 1). There was no difference in RBP4 levels between PCOS and control groups at baseline (Table 1).

Correlations with RBP4

Pearson correlations showed that RBP4 correlated with cholesterol ($r = 0.28$, $P = 0.04$) and triglycerides ($r = 0.30$, $P = 0.03$) at baseline. RBP4 did not correlate with indexes of insulin resistance, hyperandrogenism, or adiposity.

Intervention study

There were no differences in baseline characteristics for subjects with PCOS randomly assigned to metformin ($n = 19$) or the OCP ($n = 19$) (Table 2). There were no BMI changes over the study in either group.

Sex steroids. There was a time-by-treatment effect for SHBG ($P < 0.01$) and FAI ($P < 0.01$): SHBG increased with the OCP and FAI fell, compared with no change with metformin. At study completion, the OCP group had higher SHBG and lower FAI (Table 2).

Lipids and hsCRP. There was a time-by-treatment effect for HDL cholesterol ($P = 0.01$) and triglycerides ($P < 0.01$). HDL cholesterol decreased with metformin and did not change with OCP. Triglycerides increased with the OCP, but did not change with metformin. LDL cholesterol decreased with the OCP, with no time-by-treatment effect. At completion, the OCP group demonstrated higher HDL cholesterol ($P < 0.01$). There was a time-by-treatment effect for hsCRP ($P = 0.03$) with a nonsignificant reduction with metformin and a nonsignificant increase with OCP.

Insulin resistance. There was a time-by-treatment effect for AUC insulin ($P < 0.01$) with a 33% increase in AUC insulin with the OCP and a 35% decrease with metformin (Fig. 1A), and the groups were significantly different after treatment.

RBP4. Despite decreased insulin resistance with metformin and increased insulin resistance with the OCP, RBP4 levels did not change in either group. There was

Table 1—Characteristics at baseline of control subjects versus women with PCOS

Baseline characteristic	Control	PCOS	P value
n	17	38	
Age (years)	33.2 ± 1.9	34.1 ± 1.2	0.68
BMI (kg/m ²)	36.9 ± 1.4	36.8 ± 1.2	0.98
Weight (kg)	97.6 ± 4.3	99.6 ± 3.5	0.73
Waist circumference (cm)	108.7 ± 3.4	108.9 ± 2.6	0.94
Waist-to-hip ratio	0.85 ± 0.01	0.86 ± 0.01	0.47
hsCRP (mg/l)	3.3 ± 0.51	4.1 ± 0.5	0.36
Testosterone (nmol/l)	1.3 ± 0.2	2.2 ± 0.1	<0.01
SHBG (nmol/l)	40.4 ± 4.5	31.9 ± 2.5	0.08
FAI	4.5 ± 0.9	10.3 ± 1.6	<0.01
Fasting glucose (mmol/l)	4.5 ± 0.1	4.5 ± 0.1	0.88
Fasting insulin (mU/l)	10.3 ± 1.2	21.2 ± 3	0.01
Cholesterol (mmol/l)	4.8 ± 0.2	5.2 ± 0.2	0.1
HDL cholesterol (mmol/l)	1 ± 0	1.3 ± 0.1	<0.01
LDL cholesterol (mmol/l)	3.3 ± 0.2	3.3 ± 0.2	0.9
Triglycerides (mmol/l)	0.9 ± 0.1	1.4 ± 0.1	<0.01
HOMA	2.1 ± 0.3	4.5 ± 0.7	0.02
RBP4 (μg/ml)	28.9 ± 3.1	35.4 ± 4.3	0.36

Data are means ± SEM unless otherwise indicated.

no difference between groups in RBP4 after intervention (Fig. 1B).

Correlations with change in RBP4. Within the two groups, a change in RBP4 did not correlate with changes in insulin resistance, metabolic factors, or indexes of adiposity.

CONCLUSIONS—Recent comprehensive studies in mice provide convincing evidence of a link between insulin resistance and RBP4 (3). However, human data have been less consistent. PCOS is a known insulin-resistant state, and in the current human PCOS study, we have demonstrated on the basis of HOMA that women with PCOS are more insulin resistant than overweight control subjects but do not have different RBP4 levels. Furthermore, in PCOS, RBP4 levels did not correlate with other insulin resistance markers and did not change with interventions including metformin (which reduced insulin resistance) and the OCP (which increased insulin resistance). These data suggest that RBP4 is not a useful marker of insulin resistance in PCOS.

To date, human studies have failed to clarify the role of RBP4 as a marker of insulin resistance. RBP4 has been reported as high and correlated inversely with insulin sensitivity (with a hyperinsulinemic-euglycemic clamp) in subjects with IGT and type 2 diabetes and nonobese relatives of subjects with diabetes (4). These populations mostly had abnormal glucose metabolism (AGM) rather

than isolated insulin resistance and were predominately male. In one comparison of obese subjects with and without type 2 diabetes, the relationship between RBP4 and insulin resistance was not independent of BMI (4). Thus, the isolated contribution of insulin resistance cannot be determined. Recently, RBP4 correlations with insulin resistance have been inconsistent. Cho et al. (6) reported high plasma RBP4 levels in weight-matched humans with IGT and type 2 diabetes; however, RBP4 did not correlate with insulin resistance. Morbidly obese patients had high RBP4 levels compared with lean control subjects but notably also had AGM with elevated fasting glucose (5). RBP4 was not elevated in obese postmenopausal women without type 2 diabetes (8) and did not correlate with insulin resistance.

Obese women with PCOS present

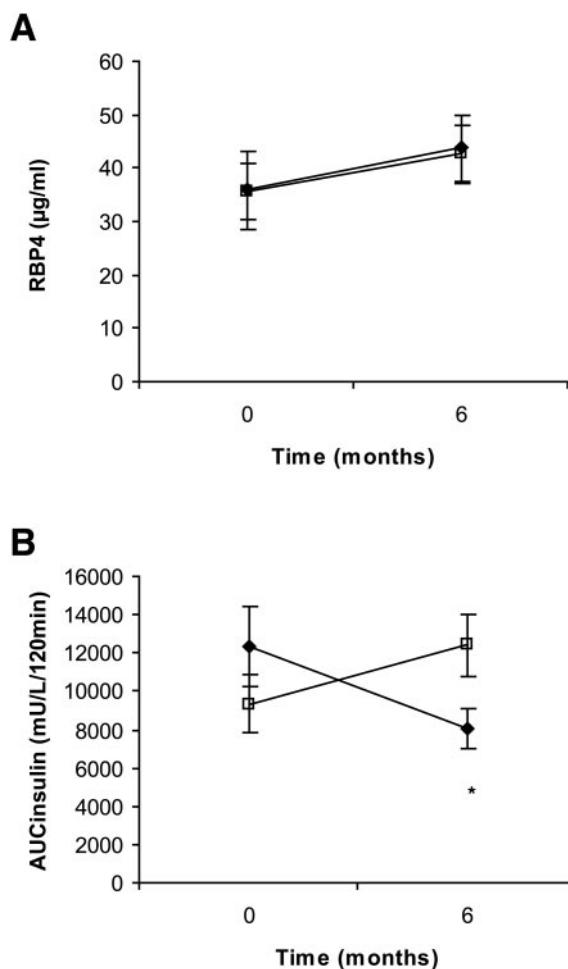


Figure 1—Changes in RBP4 (A) and AUC insulin on an OGTT (B) after metformin (◆) or the OCP (□) for 6 months. Data are means ± SEM. Data were assessed using repeated-measures ANOVA with time as the within-subject factor and intervention as the between-subject factor. *Time-by-intervention effect ($P < 0.01$) with a decrease in AUC insulin for metformin ($P = 0.03$) and increase for OCP ($P = < 0.01$)

with reproductive abnormalities before the onset of AGM and have extrinsic obesity-related insulin resistance in addition to intrinsic PCOS-related insulin resistance. They are more insulin resistant than weight-matched control subjects (2). Hence, women with PCOS provide a useful model to investigate whether RBP4 reflects insulin resistance independent of AGM. Women whose PCOS is diagnosed on the basis of the National Institutes of Health criteria demonstrate more severe insulin resistance (13,14). Although our results demonstrate a difference in insulin resistance (HOMA) between overweight women with PCOS and control subjects, RBP4 did not differ between groups. Similarly, Hahn et al. (15) did not detect a difference in RBP4 between lean women with PCOS and lean control subjects. However, in a study in 10 obese, insulin-resistant women with PCOS (7), higher RBP4 levels were seen than those in weight-matched control subjects. Of note, the enzyme-linked immunosorbent assay (ELISA) method was used rather than the Western blot. Tan et al. (7) noted that women with PCOS had a significantly higher mean fasting glucose (upper limit of normal range) than control women and that RBP4 correlated with glucose but not with HOMA or insulin levels, suggesting that RBP4 reflects AGM, not insulin resistance. This result is consistent with those in the current study with no difference in RBP4 in a population with insulin resistance but without AGM. Although PCOS is an insulin-resistant state, PCOS per se and insulin resistance do not seem to be related to high RBP4 levels.

We did not demonstrate a correlation between serum RBP4 levels and markers of insulin resistance in overweight women with PCOS at baseline, confirming previous observational PCOS studies (7,15). Other than that of Graham et al. (4), most studies in weight-matched diabetic and obese subjects have not demonstrated a relationship with RBP4 and insulin resistance (5,6,8,10). We showed a correlation with RBP4 and triglycerides, which has been noted previously (4,15). RBP4 correlates with adiposity markers or AGM relatively consistently across other studies; however, we did not demonstrate these correlations in relatively homogeneous overweight women with PCOS who had predominately normal glucose tolerance.

In the current study, despite demonstrating decreased insulin resistance after

6 months of metformin and increased insulin resistance with the OCP in overweight women with PCOS, we were unable to show any relationship between change in insulin resistance and RBP4. RBP4 has been shown to change with interventions that reduce insulin resistance in other insulin-resistant populations although results are variable. RBP4 (ELISA) decreased with 13% weight loss after lap-banding, although there was no decrease in insulin resistance (5). Similarly, no relationship was seen between RBP4 and decreased insulin resistance during weight reduction in obese women (10). Conversely, 5% weight loss did not change RBP4, despite a decrease in insulin resistance (8). In 60 subjects, exercise training improved insulin resistance (euglycemic clamp). A significant decrease in RBP4 was detected with post hoc analysis in the most insulin-resistant subjects, in whom exercise improved insulin resistance markedly (4). RBP4 levels are normalized in mice treated with the insulin sensitizer rosiglitazone. Treatment of human subjects who have IGT with the insulin sensitizer pioglitazone resulted in a paradoxical increase in RBP4 expression in adipose tissue, despite a decrease in insulin resistance. Serum RBP4 was unchanged with pioglitazone and metformin, consistent with results in the current study (9).

FAI is preferable to testosterone as a marker of androgen excess in women with PCOS. In the current study, FAI and testosterone decreased with the OCP, with no change with metformin, consistent with results in a Cochrane review (16). Two recent studies noted that testosterone and FAI decreased with metformin, yet both studies noted a decrease in BMI (17,18). The lack of changes in androgens with metformin in the current study may be related to stable BMI or to sample size.

The dyslipidemia of PCOS has both similarities with type 2 diabetes (elevated triglycerides) and differences (HDL cholesterol is not low), as noted here and in a large study by Legro et al. (19). In the current study, HDL cholesterol decreased with metformin and was unchanged with the OCP, whereas triglycerides increased with the OCP. Other studies have shown similar findings (12,17), yet a Cochrane review in PCOS (16) showed no changes in HDL cholesterol with metformin. This result contrasts with findings in type 2 diabetes, in which HDL cholesterol increases with metformin (20). These ob-

servations warrant further exploration but add to mounting evidence that PCOS is distinct from diabetes from both a reproductive and metabolic perspective.

Inconsistent results may be attributed to inaccuracies of the commercial ELISA RBP4 assays (11) and to variations in assessment of insulin resistance. Only studies using hyperinsulinemic-euglycemic clamps showed a correlation with RBP4 (4). Western blotting yields RBP4 concentrations with a greater dynamic range than the ELISA (11). The ELISA may underestimate the differences in RBP4 between insulin-sensitive and insulin-resistant subjects. Enzyme immunoassay has been shown to undervalue RBP4 concentrations, possibly because of assay saturation in insulin resistance. Some commercial ELISAs use urinary RBP4 as the protein standard rather than the full-length form found in serum, leading to greater immunoreactivity. These issues have led to the recommendation that quantitative Western blotting, standardized to full-length RBP4 protein, should be used to measure RBP4 in insulin-resistant states (11).

Insulin-resistant mice have high RBP4 in adipose tissue and serum, and these elevations can be normalized by insulin sensitizers (3). Increasing RBP4 in mice induces insulin resistance, whereas decreasing RBP4 enhances insulin sensitivity. However, RBP4 was one of five messenger RNAs encoding proteins identified in adipose tissue when DNA array analysis was performed on insulin-resistant, adipose-specific GLUT4 knockout mice (3). Work with the remaining identified proteins may elucidate the relationship between the downregulation of adipose-specific GLUT4 in humans and insulin resistance.

Strengths of our study include both a baseline and intervention phase with higher insulin resistance in PCOS at baseline, supported by intervention data with differential effects of medical therapy on insulin resistance without a change in RBP4. We have also used the Western blot technique to determine RBP4. The study was limited by a relatively small sample size. Although the accurate measurement of insulin resistance is challenging, the HOMA score and AUC insulin during an OGTT appear to be comparable to those with the hyperinsulinemic-euglycemic clamp technique in PCOS (21,22); however, clamp studies may have been more accurate.

Although women with PCOS are

more insulin resistant than overweight control subjects, this fact was not reflected by RBP4 levels. RBP4 did not correlate with other markers of insulin resistance in overweight women with and without PCOS. In addition, RBP4 did not change with changes in insulin resistance from therapeutic interventions including metformin (reduced insulin resistance) or the OCP (increased insulin resistance). RBP4 did correlate with lipid levels, suggesting that RBP4 is not a useful marker of insulin resistance but may reflect metabolic abnormalities in PCOS.

Acknowledgments— This was an investigator-initiated trial funded by a competitive CVL grant sponsored by Pfizer Australia and through a Jean Hailes Foundation Grant. Douglas Pharmaceuticals Australia provided the metformin. H.J.T. is a National Health and Medical Research Council (NHMRC) Fellow. S.K.H. Hutchison is an NHMRC PhD Scholar.

Pathological analysis was completed at Southern Health Laboratories.

References

- Diamanti-Kandarakis E, Papavassiliou AG: Molecular mechanisms of insulin resistance in polycystic ovary syndrome *Trends Mol Med* 12; 324–332, 2006
- Teede H, Hutchison SK, Zoungas S: The management of insulin resistance in polycystic ovary syndrome. *Trends Endocrinol Metab* 18:273–279, 2007
- Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB: Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes *Nature* 436:356–362, 2005
- Graham TE, Yang Q, Blüher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson P-A, Smith U, Kahn BB: Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 354:2552–2563, 2006
- Haider DG, Schindler K, Prager G, Bohdalian A, Luger A, Wolzt M, Ludvik B: Serum retinol-binding protein 4 is reduced after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab* 92:1168–1171, 2007
- Cho YM, Youn, B-S, Lee H, Lee N, Min, S-S, Kwak SH, Lee HK, Park KS: Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. *Diabetes Care* 29:2457–2461, 2006
- Tan BK, Chen J, Lehnert H, Kennedy R, Randeve HS: Raised serum, adipocyte, and adipose tissue retinol-binding protein 4 in overweight women with polycystic ovary syndrome: effects of gonadal and adrenal steroids. *J Clin Endocrinol Metab* 92:2764–2772, 2007
- Janke J, Engeli S, Boschmann M, Adams F, Böhnke J, Luft FC, Sharma AM: Jordan J: Retinol-binding protein 4 in human obesity. *Diabetes* 55:2805–2810, 2006
- Yao-Borengasser A, Varma V, Bodles AM, Rasouli N, Phanavanh B, Lee, M-J, Starks T, Kern LM, Spencer HJ III, Rashidi AA, McGehee RE Jr, Fried SK, Kern PA: Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone. *J Clin Endocrinol Metab* 92:2590–2597, 2007
- Vitkova M, Klimcakova E, Kovacicova M, Valle C, Moro C, Polak J, Hanacek J, Capel F, Viguier N, Richterova B, Bajzova M, Hejnova J, Stich V, Langin D: Plasma levels and adipose tissue messenger ribonucleic acid expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin sensitivity. *J Clin Endocrinol Metab* 92:2330–2335, 2007
- Graham TE, Wason CJ, Blüher M, Kahn BB: Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. *Diabetologia* 50:814–823, 2007
- Meyer C, McGrath BP, Teede HJ: Effects of medical therapy on insulin resistance and the cardiovascular system in polycystic ovary syndrome. *Diabetes Care* 30:471–478, 2007
- Broekmans FJ, Knauff EAH, Valkenburg O, Laven JS, Eijkemans MJ, Fauser BCJM: PCOS according to the Rotterdam consensus criteria: change in prevalence among WHO-II anovulation and association with metabolic factors. *BJOG* 113:1210–1217, 2006
- Carmina E, Chu MC, Longo RA, Rini GB, Lobo RA: Phenotypic variation in hyperandrogenic women influences the findings of abnormal metabolic and cardiovascular risk parameters. *J Clin Endocrinol Metab* 90:2545–2549, 2005
- Hahn S, Backhaus M, Broecker-Preuss M, Tan S, Dietz T, Kimmig R, Schmidt M, Mann K, Janssen OE: Retinol-binding protein 4 levels are elevated in polycystic ovary syndrome women with obesity and impaired glucose metabolism. *Eur J Endocrinol* 157:201–207, 2007
- Lord JM, Flight IH, Norman RJ: Insulin-sensitising drugs (metformin, troglitazone, rosiglitazone, pioglitazone, D-chiro-inositol) for polycystic ovary syndrome *Cochrane Database Syst Rev* CD003053, 2003
- Heutling D, Schulz H, Nickel I, Kleinsteijn J, Kaltwasser P, Westphal S, Mittermayer F, Wolzt M, Krzyzanowska K, Randeve H, Schernthaner G, Lehnert H: Asymmetrical dimethylarginine, inflammatory and metabolic parameters in women with polycystic ovary syndrome before and after metformin treatment. *J Clin Endocrinol Metab* 93:82–90, 2008
- Legro RS, Barnhart HX, Schlaff WD, Carr BR, Diamond MP, Carson SA, Steinkamp MP, Coutifaris C, McGovern PG, Cataldo NA, Gosman GG, Nestler JE, Giudice LC, Leppert PC, Myers ER, the Cooperative Multicenter Reproductive Medicine Network: Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. *N Engl J Med* 356:551–566, 2007
- Legro RS, Kunselman AR, Dunaif A: Prevalence and predictors of dyslipidemia in women with polycystic ovary syndrome. *Am J Med* 111:607–613, 2001
- Saenz A, Fernandez-Esteban I, Mataix A, Ausejo M, Roque M, Moher D: Metformin monotherapy for type 2 diabetes mellitus. *Cochrane Database Syst Rev* CD002966, 2005
- Ciampelli M, Leoni F, Cucinelli F, Mancuso S, Panunzi S, De Gaetano A, Lanzone A: Assessment of insulin sensitivity from measurements in the fasting state and during an oral glucose tolerance test in polycystic ovary syndrome and menopausal patients. *J Clin Endocrinol Metab* 90:1398–1406, 2005
- Mather KJ, Hunt AE, Steinberg HO, Paradisi G, Hook G, Katz A, Quon MJ, Baron AD: Repeatability characteristics of simple indices of insulin resistance: implications for research applications. *J Clin Endocrinol Metab* 86:5457–5464, 2001