Published in final edited form as:

Metabolism. 2009 April ; 58(4): 479–487. doi:10.1016/j.metabol.2008.11.004.

Retinol-Binding Protein Levels are Increased in Association with Gonadotropin Levels in Healthy Women

Hideo Makimura¹, Jeffrey Wei¹, Sara E. Dolan-Looby¹, Vincent Ricchiuti², and Steven Grinspoon¹

¹ Program in Nutritional Metabolism and Neuroendocrine Unit, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02114

² Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts 02115

Abstract

Recent studies have demonstrated an association between retinol-binding protein (RBP4) and insulin resistance. RBP4 is decreased in women and elevated in polycystic ovary syndrome. However, prior studies have not investigated the relationship between RBP4, gonadal steroids and gonadotropins in healthy women. The aim of this study was to determine the RBP4 levels in a cohort of healthy women with a range of body mass indices and glucose tolerances, to investigate the relationship between RBP4, gonadotropin levels and menopausal status. Serum RBP4 levels were measured by ELISA and quantitative Western blot in eighty-eight healthy women (aged 24- to 59-years old) from the general community in a cross sectional study. RBP4 was higher in post-menopausal, compared to pre-menopausal women (26.1 ± 2.1 vs. 19.3 ± 0.5 $\mu\text{g/ml}$; $p = 0.001$). In univariate analysis, RBP4 was associated with follicle stimulating hormone (FSH) ($r = 0.37$; $p = 0.0004$), luteinizing hormone (LH) ($r = 0.3$; $p = 0.005$), sex hormone binding globulin (SHBG) ($r = -0.24$; $p = 0.03$) and trended to significance with estradiol ($p = 0.09$) but not with free testosterone or dehydroepiandrosterone-sulfate. RBP4 was also associated with insulin at 2-hours during an oral glucose tolerance test (OGTT) ($r = 0.24$; $p = 0.03$) and the area under the curve (AUC) for insulin during the OGTT ($r = 0.26$; $p = 0.02$). In multivariate regression modeling, both FSH ($p = 0.03$) and LH ($p = 0.04$) remained significantly associated with RBP4 after controlling for estradiol, SHBG, insulin AUC, cholesterol, triglycerides, waist-to-hip ratio and tumor necrosis factor α . RBP4 was not associated with inflammatory markers or with carotid intima-media thickness. Therefore, RBP4 is higher in post-menopausal females and is associated with gonadotropin concentrations in healthy women.

Keywords

adipokine; FSH; LH; insulin resistance; carotid intima-media thickness

1. Introduction

Recent studies have identified a novel adipokine, retinol-binding protein (RBP4), as a putative mediator of insulin resistance (1). Both transgenic and pharmacological increases in RBP4

Corresponding Author: Hideo Makimura, MD, PhD, Program in Nutritional Metabolism, Massachusetts General Hospital and Harvard Medical School, 55 Fruit St., LON 211, Boston, MA 02114, Tel: 617-726-8277, Fax: 617-724-8998, Email: hmakimura@partners.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

result in development of insulin resistance. Conversely reductions in RBP4 levels by knock out or pharmacologic means decrease insulin resistance in mice (1). The effects of RBP4 are thought to be mediated by alterations in insulin signaling through insulin receptor substrate-1 and phosphoinositide 3-kinase activation (1). In contrast to the data in mice, the role of RBP4 in humans is less clear. Although the majority of studies support a role for RBP4 in mediating insulin resistance (2–8), several studies do not support such an effect (9–11). In addition, an association between RBP4 and abdominal obesity has been shown in some (2,7,8) but not all studies (3,5,6,9,11).

Relatively little is known regarding the potential regulation of RBP4 by sex steroids and related gonadotropins and the primary purpose of our study was to investigate the relationship of RBP4 with these parameters. While two studies have reported a lack of sexual dimorphism in RBP4 (5,10), three other studies have demonstrated lower RBP4 levels in females subjects (3,6,8). More over, serum RBP4 levels are elevated in polycystic ovary syndrome (PCOS) and associated with insulin resistance (12). Tan et al. confirmed the elevated RBP4 levels in PCOS and further demonstrated an increase in both mRNA and protein levels of RBP4 in adipose tissue explants from PCOS subjects after treatment with 17- β -estradiol in a dose dependent manner. This effect was specific to estradiol as testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEA-S) did not have any effects on RBP4 mRNA or protein levels in human adipose tissue explants (13).

Adipocytes express estrogen receptor α and β (14) as well as androgen receptors (15). Higher RBP4 levels in men as well as in PCOS suggest that androgens may increase levels of RBP4 and/or that estrogen may lower levels of RBP4. In contrast, *in vitro* data demonstrate that 17- β -estradiol directly stimulates both mRNA and protein levels of RBP4 in adipose tissue explants of PCOS subjects (13). Interestingly, adipocytes also express mRNA for pituitary hormone receptors (16) and functionally respond with activation of various signaling cascades in response to pituitary hormones (17). More recently, adipocytes were found to express receptors for luteinizing hormone (LH) and respond to human chorionic gonadotropin (HCG) with increased growth and differentiation (18). This raises the alternative hypothesis that RBP4 is regulated directly by the gonadotropins, LH and follicle stimulating hormone (FSH), independently and in addition to gonadal steroids.

We had previously demonstrated an association of another adipokine, adiponectin, to carotid intima-media thickness (IMT), a measure of atherosclerosis, in otherwise healthy women (19). Adiponectin is inversely associated with obesity (20), insulin resistance and diabetes (21). Interestingly, it is also lower in patients with coronary artery disease (22). Adiponectin is therefore thought to have an anti-atherosclerotic and anti-inflammatory effect (23,24). Similarly, two previous studies have demonstrated positive correlations between c-reactive protein (CRP) and RBP4 (7,8), also suggesting a role for RBP4 in mediating inflammation and atherosclerosis. However, no study has evaluated the relationship between RBP4 and atherosclerosis directly with carotid IMT. A secondary endpoint of the study was therefore to determine the relationship between RBP4, inflammatory indices and carotid IMT in women.

We measured serum RBP4 levels in a previously well characterized cohort of female subjects and compared RBP4 levels by menopausal status, serum FSH, LH, estradiol, testosterone and DHEA-S levels to determine the relationship between RBP4, sex steroids, and gonadotropins. In addition, RBP4 levels were analyzed for association with indices of insulin sensitivity, fat distribution, and carotid IMT. We hypothesized that RBP4 would increase in association with gonadotropin levels and be associated with carotid IMT in otherwise healthy women.

2. Subjects and Methods

2.1. Subjects

Subjects were recruited from the community at large through advertisement and primary care referrals between October 2000 and May 2004. Inclusion criteria included age between 18 and 60 years old, female gender, body mass index (BMI) greater than 20 kg/m² and less than 35 kg/m² and healthy, without known acute or chronic illnesses (including not receiving medications for diabetes, hypertension or dyslipidemia or acute infections within 3 months of study). Exclusion criteria included use of steroids, growth hormone, oral contraception pills, or any anabolic agents within 3 months of study. Other exclusion criteria included substance abuse, pregnancy or breast-feeding within the past year, or history of bilateral oophorectomy. One post-menopausal subject was taking hormone replacement therapy and had an estradiol of 36.6 pg/ml and a FSH of 21 IU/l. Data was analyzed with and without this subject and were not found to be any different. Therefore the final results presented here include data from this subject. Data from this cohort establishing the relationship between adiponectin and IMT were previously published (19). The study was approved by the Massachusetts General Hospital (MGH) and Massachusetts Institute of Technology (MIT) institutional review committees where they were seen. All subjects provided written consent.

2.2. Reproductive Function

Menstrual history was obtained in all subjects. History of hysterectomy or oophorectomy was obtained. Based on history, subjects were categorized as eumenorrheic (normal menstrual function), oligomenorrheic (less than 3 menstrual periods in the 3 months before enrollment), or amenorrheic.

2.3. Hormonal measurements

All testing was performed in the early follicular phase when appropriate (for pre-menopausal eumenorrheic subjects) after a 12-hour over night fast. Measurements were obtained in duplicate and average values are reported. Serum RBP4 was measured using an ELISA (ALPCO Diagnostics, Salem, NH) and quantitative Western blots. Intra-assay coefficient of variation (CVs) for ELISA was 5% and inter-assay CV ranged from 9.7 to 9.8%. Details on the Western blot methodology are presented below. Serum FSH was measured using a solid-phase immunoradiometric assay (Diagnostic Products Corp., Los Angeles, CA) with an intra-assay CV of 2.2 to 3.8%. Serum LH was also measured using a solid-phase immunoradiometric assay (Diagnostic Products Corporation, Los Angeles, CA) with an intra-assay CV of 1.0 to 1.6%. Serum estradiol was measured by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX) with an intra-assay CV of 6.5 to 8.9%. Serum free testosterone concentration was determined as the product of the percent free testosterone concentration, measured by equilibrium dialysis, and the total testosterone concentration (Endocrine Sciences, Inc., Calabasas Hills, CA). The intra-assay CV of free testosterone was 6.9% and total testosterone was < 8.1%. The sensitivity of the percent free testosterone assay was 0.1% and the sensitivity of the total testosterone assay was 3 ng/dl. Sex hormone binding globulin (SHBG) levels were measured by immunoradiometric assay (Esoterix, Austin, TX) with an intra-assay CV of < 4% and an inter-assay CV of 7.8 to 10.6%. Serum DHEA-S was measured by RIA (Siemens Medical Solutions Diagnostics, Los Angeles, CA) with an intra-assay CV range of 3.8 to 5.3% and inter-assay CV range of 6.3 to 11%. Insulin and glucose were measured at 0, 30, 60, 90 and 120 minutes after a standard 75-g oral glucose challenge (OGTT). Serum insulin levels were measured using a RIA (Diagnostic Products Corp., Los Angeles, CA) with intra-assay and inter-assay CV range from 3.1 to 9.3% and 4.9 to 10% respectively. Adiponectin was measured using a RIA (Linco Research, St. Charles, MO) with an intra-assay and inter-assay CV range of 1.8 to 6.2% and 6.9 to 9.3% respectively. CRP was measured using an ultra-sensitive ELISA (Diagnostic System Laboratories, Webster, TX) with an intra-assay and inter-

assay CV range of 1.7 to 3.9% and 2.8 to 5.1%, respectively. The sensitivity (minimum detection limit) of the assay is 1.6 ng/ml. Homocysteine was measured by recombinant enzymatic cycling assay (Carolina Liquid Chemistries, Brea, CA) with an intra-assay and inter-assay CV range of 1.8 to 3 % and 1 to 3 %, respectively. Levels of both Tumor Necrosis Factor α (TNF α) and interleukin-6 (IL-6) were measured using ELISA kits from R&D Systems (Minneapolis, MN). The ELISA for TNF α has an intra-assay and inter-assay CV range of 4.2 to 5.6% and 4.6 to 7.4%, respectively while the ELISA for IL-6 has an intra-assay and inter-assay CV range of 6.9 to 7.8% and 6.5 to 9.6%, respectively.

2.4. Western blot

Quantitative Western blotting using full-length recombinant Retinol Binding Protein 4 (rRBP4) (3378-LC, R&D Systems, Minneapolis, MN) was validated and calibration curve using rRBP4 (standards solution) was performed in each Western blot along with samples. Samples were analyzed in duplicate and average OD values were used to determine the quantity. Standard solutions of 15, 30, 60 or 120 μ g/ml rRBP4 were prepared in Tris-buffered saline containing 1% NP-40. Standards and sera were diluted 1:100 in SDS-PAGE buffer and boiled for 5 min. 15 μ l of diluted standards or sera and molecular weight markers were electrophoresed on 18% Tris-glycine SDS-PAGE gels (Criterion brand; BioRad) and transferred to nitrocellulose. Western blotting was performed with non-fat milk (BioRad) as a blocking agent. Blots were incubated overnight at 4°C with primary antibody directed against RBP4 (A0040, DAKOCytomation, DAKO USA, Carpinteria, CA) diluted 1:1,000 and for 1 hour at room temperature with horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:2,000). Bands were detected by enzymatic chemiluminescence (GE Healthcare Bio-Sciences, Piscataway, NJ) and quantified with ImageJ software (NIH, Bethesda, MD). A single band for rRBP4 migrated at about 21 kDa. Linear curve was used to fit the purified RBP4 standards ($r^2 > 0.98$) for each individual gel and to calculate concentrations in serum samples. This method provides absolute measurements of RBP4 concentrations with minimal gel to gel variability ($\pm 10\%$ CV for the same sample from duplicate gels). To avoid irregularities from gel quality or protein transfer, RBP4 standards were placed in the same position on each gel. To monitor assay quality, serum reference samples were included on every gel.

2.5. Carotid IMT

Carotid intima-media thickness (IMT) measurements were performed as previously described (19,25). Briefly, imaging was conducted using a high-resolution 7.5 MHz phased array transducer (Hewlett Packard SONOS 2000/2500). Digital images were captured directly to a Windows NT workstation using a high-quality, high-speed frame capture card made by Data Translation (Marlboro, MA). Either the 90 or the 45-degree image in diastole was selected as the best view for image quality. Edge detection and mean IMT calculation were accomplished with an in house computer program. The published reproducibility of the technique is excellent with a standard deviation of 0.007 mm (26). The average IMT over the length of the measured segments on the right is reported.

2.6. Body Composition

Weight and anthropometric measurements were determined in the morning, prior to breakfast. Anthropometric measurements were obtained using an inelastic tape measure by the Bionutrition Staff of the MIT General Clinical Research Center. Cross-sectional abdominal CT scans at the level of the L4 pedicle were performed to assess abdominal visceral and subcutaneous adipose tissue area (VAT and SAT, respectively) as previously reported (19, 25).

2.7. Statistical Methods

Continuous variables were tested for normality of distribution with the use of the Wilk-Shapiro test and examination of the histogram distribution. Variables that were normally distributed were compared using the student's t-test while those variables that were not normally distributed were compared using the non-parametric Wilcoxon rank sum test. Statistical significance was assumed when the null hypothesis could be rejected at $p < 0.05$. Univariate regression analysis was performed comparing RBP4 and other variables using the Pearson correlation coefficient. A standard least-squares multivariate regression analysis was performed. All variables that were significant on univariate analysis with a $p < 0.1$ were included in the analysis. In addition, a step-wise forward-selection multivariate regression analysis was performed, with $p < 0.1$, to enter, to confirm the results of the multivariate analysis. LH and FSH were entered as continuous variables in these analyses. All statistical analyses were performed using SAS JMP statistical software (version 5.1; SAS Institute Inc., Cary, NC).

3. Results

3.1. Characteristics of the Study Subjects

Eighty-eight otherwise healthy women aged 24- to 59-years old were enrolled in this study and their characteristics described in Table 1. Eleven subjects were post-menopausal as determined by follicular phase FSH > 20 IU/l and seventy-seven were categorized as pre-menopausal. None of the subjects with FSH > 20 IU/l were under the age of 40.

3.2. RBP4 Concentrations in Menopausal Women

Menopausal subjects were older compared to pre-menopausal subjects (49.3 ± 1.6 vs. 39.6 ± 0.7 years; $p < 0.0001$). Both FSH (83.6 ± 15.6 vs. 6.2 ± 0.4 IU/L; $p < 0.0001$) and LH (27.1 ± 4.7 vs. 5.1 ± 0.6 IU/L; $p < 0.0001$) were higher and estradiol (20.3 ± 5.4 vs. 58.6 ± 5.4 pg/ml; $p = 0.0004$), free testosterone levels as determined by equilibrium dialysis technique (1.5 ± 0.3 vs. 3 ± 0.2 pg/ml; $p = 0.002$) and DHEA-S (84.7 ± 17.1 vs. 135.3 ± 11.9 µg/dl; $p = 0.04$) were lower in the post-menopausal women. Insulin area under the curve (AUC) during the oral glucose tolerance test (OGTT) was higher in the post-menopausal subjects (6153.7 ± 527.3 vs. 5201.8 ± 279.4 ; $p = 0.04$), however fasting insulin and 2 hour insulin during the OGTT were not significantly different. There were no significant differences between bodyweight, indices of body fat distribution or inflammatory markers (TNF- α , IL-6, homocysteine, CRP). Total cholesterol (199.9 ± 13.4 vs. 173.4 ± 3.4 mg/dl; $p = 0.03$) and HDL cholesterol (70.3 ± 4 vs. 55.6 ± 1.6 mg/dl; $p = 0.002$) were higher in the post-menopausal group, while triglycerides and LDL cholesterol were not different. Resting energy expenditure was lower (1271.8 ± 45.7 vs. 1410.3 ± 21.4 kcal; $p = 0.02$) in the post-menopausal group. Both adiponectin (10.2 ± 1.2 vs. 8.1 ± 0.7 µg/ml; $p = 0.03$) and carotid IMT were higher (0.7 ± 0.03 vs. 0.63 ± 0.01 mm; $p = 0.007$) in the post-menopausal group (Table 1). Serum RBP4 levels were 35% higher in post-menopausal women (26.1 ± 2.1 vs. 19.3 ± 0.5 µg/ml; $p = 0.001$) (Figure 1).

3.3 Confirmation of RBP4 Results by Quantitative Western Blot

Results for RBP4 were recapitulated using quantitative Western Blot analysis. RBP4 concentrations assessed by ELISA correlated significantly with RBP4 by Western Blot ($r = 0.42$; $p < 0.0001$). Moreover, differences by menstrual status in RBP4 concentrations were also confirmed using Western Blot, e.g. RBP4 levels were higher in post-menopausal than pre-menopausal subjects (96.5 ± 12.8 vs. 75.9 ± 4.8 µg/ml; $p < 0.05$).

3.4 Univariate and Multivariate Regression Analyses

The results of the univariate and multivariate regression analyses are summarized in Tables 2 and 3, respectively. Among the entire group, including pre- and post-menopausal subjects,

RBP4 was positively associated with FSH ($r = 0.37$; $p = 0.0004$) and LH ($r = 0.3$; $p = 0.005$) and negatively associated with SHBG ($r = -0.24$; $p = 0.03$). The association between RBP4 and estradiol tended toward significance ($r = -0.18$; $p = 0.09$). In contrast RBP4 was not associated with free testosterone or DHEA-S. RBP4 was positively associated with 2-hour insulin ($r = 0.24$; $p = 0.03$) and AUC for insulin during OGTT ($r = 0.26$; $p = 0.02$) and trended to significance with fasting insulin ($r = 0.2$; $p = 0.07$). In contrast, RBP4 was not associated with fasting glucose, 2-hour glucose during OGTT, age, body weight or indices of body fat distribution. RBP4 was not associated with adiponectin, IL-6, homocysteine, or CRP, and trended to significance with TNF α ($r = -0.21$; $p = 0.08$). RBP4 was not associated with carotid IMT (Table 2).

A standard least-squares multiple regression analysis was performed with variables which reached a $p < 0.1$ on univariate analysis. These variables included FSH, estradiol, SHBG, insulin AUC, waist-to-hip ratio, total cholesterol, triglycerides and TNF α . LH was initially excluded as it was co-linear with FSH and less strongly related to RBP4 on univariate analysis. Similarly, fasting insulin and 2-hour insulin during OGTT were excluded as they were co-linear with and less strongly related to RBP4 than insulin AUC in the univariate analysis. The total R^2 for the model was 0.3 with a $p < 0.01$. FSH remained significantly related to RBP4 ($\beta = 0.04$; $p = 0.03$) after controlling for these parameters (Table 3A). Step wise forward regression modeling also identified FSH as most robustly related to RBP4.

Creation of a standard least-squares model with LH instead of FSH was also significant with a total R^2 for the model of 0.29 and a $p = 0.01$. LH remained significantly related to RBP4 ($\beta = 0.12$; $p = 0.04$) after controlling for the parameters as above (Table 3B). Step wise forward regression modeling also identified LH as most robustly related to RBP4. However, the addition of both LH and FSH to the model resulted in loss of significance between RBP4 and either gonadotropin, confirming co-linearity.

As RBP4 is made in adipocytes, models adjusting for body weight and indices of body fat distribution were also tested. None of the more specific indices of body fat, e.g. VAT were significant in the multivariate regression modeling. A model was also created with age given its association with menopause but was also not significant. In addition, further modeling controlling for physical activity, alcohol and tobacco use, separately and together, were also created without significant change in the overall findings. The significant relationship between RBP4 and gonadotropins seen in the primary model were confirmed in all subsequent models, none of which resulted in a significantly greater R^2 than the primary model.

4. Discussion

In this study, we demonstrate a novel relationship between gonadotropins and RBP4, a putative mediator of insulin resistance, in healthy women. Serum RBP4 levels were higher in post-menopausal subjects compared to pre-menopausal subjects and were significantly related to FSH and LH on univariate analysis in our cohort of healthy women. The relationship between gonadotropins and RBP4 remained significant on multivariate regression analysis controlling for estradiol, body weight, body fat distribution, and measures of insulin sensitivity as well as physical activity, tobacco and alcohol use, suggesting an independent relationship between gonadotropins and RBP4. In addition, RBP4 was not associated with any inflammatory markers including TNF α , IL6, homocysteine, CRP, or adiponectin and furthermore, RBP4 was not associated with carotid IMT, although our study may have been underpowered to detect these secondary end points.

Serum RBP4 concentrations determined in our study were within the previously published range for subjects without glucose intolerance or type II diabetes (2,5). Consistent with its

putative role as a mediator of insulin resistance (2–6), we observed a significant relationship between RBP4 and 2-hour insulin as well as insulin AUC during OGTT and thus our results extends this observation to healthy women. Consistent with previous data (3,5,6,9,11), we were unable to detect a significant effect of body weight, BMI, percent body fat or body fat distribution on RBP4 levels in healthy women, not selected for obesity or insulin resistance. As RBP4 is primarily made in the liver (27), the degree of liver fat may be a more prominent predictor of RBP4 levels rather than measures of generalized adiposity as demonstrated by Stefan et al. (5). We did not assess for other conditions, including sleep apnea, which might also affect insulin resistance and RBP4 concentrations.

The most striking finding of this study is the relationship between RBP4 and gonadotropin concentrations. RBP4 was found to be significantly elevated in post-menopausal women compared to pre-menopausal women. In univariate regression analysis of both pre- and post-menopausal women, both FSH and LH were significantly associated with RBP4 and this association remained significant after controlling for other variables including age, physical activity, tobacco use, alcohol use, body weight, indices of body fat distribution and insulin sensitivity, cholesterol, SHBG and estradiol in multivariate regression analysis. Our data therefore suggests an independent relationship between gonadotropins and RBP4. A potential role for gonadal steroids in the regulation of RBP4 was recently illustrated by Tan et al. (13). Tan et al., treated omental and subcutaneous adipose tissue explants from PCOS subjects with 17- β -estradiol and noted an increases in RBP4 mRNA at 4-hours and RBP4 protein levels at 24-hours. No effects were seen on RBP4 mRNA or protein levels after treatment with testosterone, androstenedione, DHEA-S or insulin. These findings are consistent with a direct role for 17- β -estradiol in the regulation of RBP4. However, in our post-menopausal women, estradiol levels are lower, suggesting that the increase in RBP4 demonstrated in post-menopausal women is unlikely to be due to estradiol. In contrast, our data suggest a potential role for gonadotropins, independent of gonadal steroids, in the regulation of RBP4.

The physiological significance of a 35% difference in RBP4 between pre- and post-menopausal women is not clear. In animal models, such as heterozygous Rbp4 knock out (Rbp4^{+/-}) mice, physiological sequelae including improvement in insulin sensitivity with increased PI3Kinase activity was noted with a 50% reduction in serum levels of RBP4 (1). In previously performed cross sectional studies of humans, other studies have found even smaller differences between groups ranging from 15 % increase in diabetic subjects compared to normal glucose tolerant subjects (3) to 23 % increase in serum RBP4 levels in obese compared to non-obese adolescents (8). In an interventional study of human subjects, a 43 % reduction in RBP4 levels was associated with improved insulin sensitivity by exercise training (2). Therefore, a 35% difference in an abundantly circulating protein may be clinically significant.

The strong relationship seen between RBP4 and gonadotropins may also contribute to the changes in insulin sensitivity associated with menopause (28), e.g. RBP4 may mediate the insulin resistance seen with aging in women. Insulin AUC during the OGTT were higher in the post-menopausal subjects, and RBP4 did correlate with insulin parameters among the entire group of pre- and post-menopausal women in our study. These data obtained in women support the relationship of RBP4 to glucose metabolism previously reported in men (2,4), and suggest that this relationship exists in women, despite sexual dimorphism in circulating RBP4 concentrations.

It is not clear from our study whether FSH, LH, or both might potentially regulate RBP4. In our analysis, FSH had a slightly stronger statistical association with RBP4, but the association with both FSH and LH were very strong, and thus it is not clear if one or both might potentially regulate RBP4. To date, only LH receptors have been identified on adipocytes (18), suggesting LH may be the physiological regulator of RBP4 rather than FSH. Alternatively, RBP4 may be

regulated by covariates or downstream elements of LH/FSH. Conversely, RBP4 may modulate gonadotropin secretion through effects on gonadal steroid regulation or an as yet unknown mechanism. Causality can not be determined from a cross-sectional study, and it will be important to determine the direct effects of FSH, LH and estradiol on RBP4 in prospective studies of women.

The hypothesis that RBP4 is regulated by gonadotropins and/or gonadal steroids is not necessarily contrary to the sexual dimorphism noted in previous reports and may in fact explain some of the heterogeneity in the previous data. Both gonadotropins and gonadal steroid levels vary through the menstrual cycle and after menopause. Therefore the timing of sampling from female subjects may have biased the previously reported RBP4 values in the female population. In addition, as menopausal status is associated with age, changes in body composition and body fat distribution (29,30), insulin resistance (28) and the metabolic syndrome (31), it is not inconceivable that previous studies of RBP4 may have been confounded by this relationship. Lastly, RBP4 is known to be made primarily in the liver followed by adipocytes (27) and hepatic and extra-hepatic production of RBP4 may also be confounding the sexual dimorphism of RBP4.

RBP4 was not associated with adiponectin in this cohort of healthy women consistent with findings from previous studies (5,10). The lack of association is suggestive of a different physiologic regulatory mechanism and function. Inflammation has been postulated to play a role in mediating the manifestation of metabolic complications of obesity and particularly that of insulin resistance (32). However, the association between inflammatory indices and RBP4 has yet to be clearly demonstrated. Two studies have demonstrated positive correlations between CRP and RBP4 (7,8), and one study has failed to detect this association (10). We did not detect a significant correlation with inflammatory markers including IL6, homocysteine or CRP and RBP4, although a trend toward significance with TNF α was noted. Moreover, there was no relationship between RBP4 and carotid IMT suggesting RBP4 is not involved in the pro-inflammatory, pro-atherosclerotic state associated with obesity, contrary to adiponectin (23,24). However, these results should be interpreted with some caution given the lack of sufficient power inherent in our unequal sample size and will need to be confirmed in future studies.

We confirmed our results from ELISA using quantitative Western blotting as Graham et al. suggest that quantitative Western blotting is the most reliable method of assaying serum RBP4 (33). The values reported by ELISA were on the average lower than by Western blot, consistent with prior reports in the literature demonstrating a greater dynamic range for the Western blot (33). The ELISA and Western blot assays demonstrated a strong positive correlation ($p < 0.0001$). Moreover, we confirmed the primary results, e.g. that RBP4 levels are higher in post-menopausal women using the Western blot.

Our study has a number of limitations. Causality or the directionality of the relationship between gonadotropins and RBP4 can not be determined from a cross sectional study, nor can we exclude effects of a parameter which may covary with FSH or LH, e.g. inhibin. Our sample is also admittedly skewed with more pre-menopausal subjects than post-menopausal subjects which could lead to lack of sufficient power to detect true differences. We were nonetheless able to detect a significant difference between RBP4 in pre- and post-menopausal subjects, and this difference is supported by the strong relationship between RBP4 and gonadotropins seen in the regression analyses.

In summary, we conclude that RBP4 is higher in post-menopausal women and associated with gonadotropins in healthy women. The strong association with insulin during OGTT confirms prior work suggesting a relationship between RBP4 and insulin resistance and extends this

observation to healthy women. In addition, RBP4 was not associated with any indices of inflammation or with carotid IMT. Further studies are necessary to delineate the physiological relationship between gonadotropins and RBP4 and to confirm the lack of relationship between RBP4, inflammatory markers and cardiovascular disease risk in women.

References

1. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 2005;436(7049):356–62. [PubMed: 16034410]
2. Graham TE, Yang Q, Bluher M, Hammarstedt A, Ciaraldi TP, Henry RR, et al. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 2006;354(24):2552–63. [PubMed: 16775236]
3. Cho YM, Youn BS, Lee H, Lee N, Min SS, Kwak SH, 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(11):2457–61. [PubMed: 17065684]
4. Gavi S, Stuart LM, Kelly P, Melendez MM, Mynarcik DC, Gelato MC, et al. Retinol-binding protein 4 is associated with insulin resistance and body fat distribution in nonobese subjects without type 2 diabetes. *J Clin Endocrinol Metab* 2007;92(5):1886–90. [PubMed: 17299074]
5. Stefan N, Hennige AM, Staiger H, Machann J, Schick F, Schleicher E, et al. High circulating retinol-binding protein 4 is associated with elevated liver fat but not with total, subcutaneous, visceral, or intramyocellular fat in humans. *Diabetes Care* 2007;30(5):1173–8. [PubMed: 17259477]
6. Jia W, Wu H, Bao Y, Wang C, Lu J, Zhu J, et al. Association of serum retinol-binding protein 4 and visceral adiposity in Chinese subjects with and without type 2 diabetes. *J Clin Endocrinol Metab* 2007;92(8):3224–9. [PubMed: 17550959]
7. Balagopal P, Graham TE, Kahn BB, Altomare A, Funanage V, George D. Reduction of elevated serum retinol binding protein in obese children by lifestyle intervention: association with subclinical inflammation. *J Clin Endocrinol Metab* 2007;92(5):1971–4. [PubMed: 17341558]
8. Lee DC, Lee JW, Im JA. Association of serum retinol binding protein 4 and insulin resistance in apparently healthy adolescents. *Metabolism* 2007;56(3):327–31. [PubMed: 17292720]
9. Janke J, Engeli S, Boschmann M, Adams F, Bohnke J, Luft FC, et al. Retinol-binding protein 4 in human obesity. *Diabetes* 2006;55(10):2805–10. [PubMed: 17003346]
10. Takebayashi K, Suetsugu M, Wakabayashi S, Aso Y, Inukai T. Retinol binding protein-4 levels and clinical features of type 2 diabetes patients. *J Clin Endocrinol Metab* 2007;92(7):2712–9. [PubMed: 17440021]
11. Yao-Borengasser A, Varma V, Bodles AM, Rasouli N, Phanavanh B, Lee MJ, et al. Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone. *J Clin Endocrinol Metab* 2007;92(7):2590–7. [PubMed: 17595259]
12. Weiping L, Qingfeng C, Shikun M, Xiurong L, Hua Q, Xiaoshu B, et al. Elevated serum RBP4 is associated with insulin resistance in women with polycystic ovary syndrome. *Endocrine* 2006;30(3):283–7. [PubMed: 17526940]
13. Tan BK, Chen J, Lehnert H, Kennedy R, Randeva 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* 2007;92(7):2764–72. [PubMed: 17456573]
14. Pedersen SB, Bruun JM, Hube F, Kristensen K, Hauner H, Richelsen B. Demonstration of estrogen receptor subtypes alpha and beta in human adipose tissue: influences of adipose cell differentiation and fat depot localization. *Mol Cell Endocrinol* 2001;182(1):27–37. [PubMed: 11500236]
15. Dieudonne MN, Pecquery R, Boumediene A, Leneuve MC, Giudicelli Y. Androgen receptors in human preadipocytes and adipocytes: regional specificities and regulation by sex steroids. *Am J Physiol* 1998;274(6 Pt 1):C1645–52. [PubMed: 9611130]
16. Schaffler A, Binart N, Scholmerich J, Buchler C. Hypothesis paper Brain talks with fat--evidence for a hypothalamic-pituitary-adipose axis? *Neuropeptides* 2005;39(4):363–7. [PubMed: 16040119]

17. Schaffler A, Scholmerich J, Buechler C. The role of 'adipotropins' and the clinical importance of a potential hypothalamic-pituitary-adipose axis. *Nat Clin Pract Endocrinol Metab* 2006;2(7):374–83. [PubMed: 16932320]
18. Dos Santos E, Dieudonne MN, Leneuve MC, Pecquery R, Serazin V, Giudicelli Y. In vitro effects of chorionic gonadotropin hormone on human adipose development. *J Endocrinol* 2007;194(2):313–25. [PubMed: 17641281]
19. Lo J, Dolan SE, Kanter JR, Hemphill LC, Connelly JM, Lees RS, et al. Effects of obesity, body composition, and adiponectin on carotid intima-media thickness in healthy women. *J Clin Endocrinol Metab* 2006;91(5):1677–82. [PubMed: 16522690]
20. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, et al. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 2001;86(5):1930–5. [PubMed: 11344187]
21. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, et al. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 2000;20(6):1595–9. [PubMed: 10845877]
22. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, et al. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol* 2003;23(1):85–9. [PubMed: 12524229]
23. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, et al. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 2000;102(11):1296–301. [PubMed: 10982546]
24. Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, et al. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 2001;103(8):1057–63. [PubMed: 11222466]
25. Johnsen S, Dolan SE, Fitch KV, Kanter JR, Hemphill LC, Connelly JM, et al. Carotid Intimal Medial Thickness in HIV Infected Women: Effects of Protease Inhibitor Use, Cardiac Risk Factors and the Metabolic Syndrome. *J Clin Endocrinol Metab* 2006;91(12):4916–4924. [PubMed: 17003092]
26. Chan R, Kaufhold J, Hemphill LC, Lees RS, Karl WC. Anisotropic Edge-Preserving Smoothing in Carotid B-mod Ultrasound for Improved Segmentation and Intima-Media Thickness (IMT) Measurement. *Computers in Cardiology* 2000;27:37–40.
27. Soprano DR, Soprano KJ, Goodman DS. Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. *J Lipid Res* 1986;27(2):166–71. [PubMed: 3754273]
28. Lindheim SR, Buchanan TA, Duffy DM, Vijod MA, Kojima T, Stanczyk FZ, et al. Comparison of estimates of insulin sensitivity in pre- and postmenopausal women using the insulin tolerance test and the frequently sampled intravenous glucose tolerance test. *J Soc Gynecol Investig* 1994;1(2):150–4.
29. Kanaley JA, Sames C, Swisher L, Swick AG, Ploutz-Snyder LL, Steppan CM, et al. Abdominal fat distribution in pre- and postmenopausal women: The impact of physical activity, age, and menopausal status. *Metabolism* 2001;50(8):976–82. [PubMed: 11474488]
30. Toth MJ, Tchernof A, Sites CK, Poehlman ET. Effect of menopausal status on body composition and abdominal fat distribution. *Int J Obes Relat Metab Disord* 2000;24(2):226–31. [PubMed: 10702775]
31. Carr MC. The emergence of the metabolic syndrome with menopause. *J Clin Endocrinol Metab* 2003;88(6):2404–11. [PubMed: 12788835]
32. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;444(7121):860–7. [PubMed: 17167474]
33. Graham TE, Wason CJ, Bluher M, Kahn BB. Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. *Diabetologia* 2007;50(4):814–23. [PubMed: 17294166]

Figure. Serum RBP4 levels in pre- and post-menopausal women. The white bar denotes pre-menopausal women (n = 77) and the black bar denotes post-menopausal women (n = 11). Data are expressed as mean $\pm$ SEM in $\mu\text{g}/\text{mL}$. $P = 0.001$ by Wilcoxon Rank Sum test.

Table 1
Baseline demographic and clinical characteristics of the study population. Data is presented as mean $\pm$ standard error of the mean.

	All subjects (n = 88)	Pre-Menopausal (n = 77)	Post-Menopausal (n = 11)	P
Demographics				
Age (yr)	40.8 $\pm$ 0.7	39.6 $\pm$ 0.7	49.3 $\pm$ 1.6	<0.0001 [†]
Race (%)				0.22 [*]
Caucasian	33 (38)	27 (35)	6 (55)	
African American	37 (42)	33 (43)	4 (36)	
Hispanic	12 (14)	12 (16)	0 (0)	
Asian	4 (4)	4 (5)	0 (0)	
Other	2 (2)	1 (1)	1 (9)	
Anthropometric Parameters				
Weight (kg)	73.2 $\pm$ 1.6	73.9 $\pm$ 1.7	68.2 $\pm$ 3.9	0.23
Body mass index (kg/m ²)	27.1 $\pm$ 0.5	27.3 $\pm$ 0.5	26.1 $\pm$ 1.1	0.42
Iliac waist (cm)	87.8 $\pm$ 1.3	88.1 $\pm$ 1.4	85.9 $\pm$ 3.3	0.64
Hip (cm)	105.9 $\pm$ 1.0	106.1 $\pm$ 1.1	104.3 $\pm$ 2.7	0.37
Waist to hip ratio	0.83 $\pm$ 0.01	0.83 $\pm$ 0.01	0.82 $\pm$ 0.02	0.90
SAT (cm ²)	284.3 $\pm$ 13.6	286.8 $\pm$ 14.3	267.0 $\pm$ 43.2	0.52
VAT (cm ²)	72.3 $\pm$ 4.9	72.9 $\pm$ 5.5	68.5 $\pm$ 9.6	0.76
% Body fat	34.0 $\pm$ 0.7	33.9 $\pm$ 0.7	35.2 $\pm$ 1.8	0.53
Hemodynamic parameters				
Systolic blood pressure (mmHg)	110.9 $\pm$ 1.5	111.2 $\pm$ 1.6	108.6 $\pm$ 3.7	0.57
Diastolic blood pressure (mmHg)	68.5 $\pm$ 1.1	68.5 $\pm$ 1.2	68.0 $\pm$ 2.2	0.87
Endocrine Parameters				
FSH (IU/l)	15.9 $\pm$ 3.3	6.2 $\pm$ 0.4	83.6 $\pm$ 15.6	<0.0001 [‡]
LH (IU/l)	7.9 $\pm$ 1.1	5.1 $\pm$ 0.6	27.1 $\pm$ 4.7	<0.0001 [‡]
Estradiol (pg/ml)	53.8 $\pm$ 5.0	58.6 $\pm$ 5.4	20.3 $\pm$ 5.4	0.0004 [‡]
SHBG (nmol/dl)	83.0 $\pm$ 4.4	83.2 $\pm$ 4.8	81.5 $\pm$ 12.3	0.76
DHEAS (μ g/dl)	128.6 $\pm$ 10.7	135.3 $\pm$ 11.9	84.7 $\pm$ 17.1	0.04 [‡]
Serum Free Testosterone (pg/ml)	2.8 $\pm$ 0.2	3.0 $\pm$ 0.2	1.5 $\pm$ 0.3	0.002 [‡]
Metabolic Parameters				
Fasting glucose on OGTT (mg/dl)	83.8 $\pm$ 1.2	83.9 $\pm$ 1.4	82.9 $\pm$ 2.0	1.00
2-hour glucose on OGTT (mg/dl)	106.9 $\pm$ 3.6	107.3 $\pm$ 4.0	104.2 $\pm$ 7.1	0.88
Fasting insulin on OGTT (μ U/ml)	6.6 $\pm$ 0.3	6.6 $\pm$ 0.4	7.0 $\pm$ 0.4	0.10
2-hour insulin on OGTT (μ U/ml)	42.8 $\pm$ 3.6	40.8 $\pm$ 3.7	57.3 $\pm$ 12.7	0.13
Insulin AUC during OGTT	5306.3 $\pm$ 256.7	5201.8 $\pm$ 279.4	6153.7 $\pm$ 527.3	0.04 [‡]
RBP4 (μ g/ml)	20.1 $\pm$ 0.6	19.3 $\pm$ 0.5	26.1 $\pm$ 2.1	0.001 [‡]
Lipid Panel				
Total cholesterol (mg/dl)	176.7 $\pm$ 3.5	173.4 $\pm$ 3.4	199.9 $\pm$ 13.4	0.03 [‡]
HDL-cholesterol (mg/dl)	57.5 $\pm$ 1.6	55.6 $\pm$ 1.6	70.3 $\pm$ 4.0	0.002 [‡]
LDL-cholesterol (mg/dl)	104.4 $\pm$ 3.3	103.1 $\pm$ 3.3	113.5 $\pm$ 13.0	0.63
Triglycerides (mg/dl)	75.4 $\pm$ 3.9	74.7 $\pm$ 4.3	80.3 $\pm$ 9.3	0.38
Inflammatory Parameters				

	All subjects (n = 88)	Pre-Menopausal (n = 77)	Post-Menopausal (n = 11)	P
TNF α (pg/ml)	23.2 $\pm$ 4.0	24.3 $\pm$ 4.4	14.9 $\pm$ 7.1	0.30
IL6 (pg/ml)	1.9 $\pm$ 0.1	1.8 $\pm$ 0.2	2.2 $\pm$ 0.5	0.63
Homocysteine (μ mol/l)	7.2 $\pm$ 0.2	7.1 $\pm$ 0.2	7.8 $\pm$ 0.9	0.54
CRP (mg/l)	2.4 $\pm$ 0.4	2.1 $\pm$ 0.3	4.4 $\pm$ 3.1	0.52
Adiponectin (μ g/ml)	8.4 $\pm$ 0.6	8.1 $\pm$ 0.7	10.2 $\pm$ 1.2	0.03 [‡]
REE (kcal)	1393.0 $\pm$ 20.1	1410.3 $\pm$ 21.4	1271.8 $\pm$ 45.7	0.02 [‡]
Carotid IMT (mm)	0.64 $\pm$ 0.01	0.63 $\pm$ 0.01	0.70 $\pm$ 0.03	0.007 [‡]

[‡] P-value obtained from Student's t-test comparison between pre- and post-menopausal women in normally distributed samples.

[‡] P-value obtained from Wilcoxon-Rank Sum test comparison between pre- and post-menopausal women in samples which were not normally distributed.

* Race was evaluated as a dichotomous variable (Caucasian or not) using a Chi squares test.

Table 2

Univariate analysis of correlation with serum RBP4 levels ($\mu\text{g/ml}$) expressed as a Pearson Correlation coefficient (r) in all subjects including pre- and post-menopausal women ($n = 88$)

Variable	Pearson Correlation Coefficient (r)	P value
Age (yr)	0.12	0.26
Waist-to-hip ratio	0.18	0.09
Endocrine Parameters		
FSH (IU/l)	0.37	0.0004
LH (IU/l)	0.30	0.005
SHBG (nmol/dl)	-0.24	0.03
Estradiol (pg/ml)	-0.18	0.09
Serum Free Testosterone (pg/ml)	-0.02	0.88
DHEAS ($\mu\text{g/dl}$)	-0.01	0.90
Metabolic Parameters		
Fasting Insulin on OGTT ($\mu\text{U/ml}$)	0.2	0.07
2-hr Insulin on OGTT ($\mu\text{U/ml}$)	0.24	0.03
Insulin AUC	0.26	0.02
Fasting Glucose on OGTT (mg/dl)	-0.08	0.44
2-hr Glucose on OGTT (mg/dl)	-0.06	0.60
Lipid Panel		
Total Cholesterol (mg/dl)	0.24	0.02
HDL (mg/dl)	0.04	0.73
LDL (mg/dl)	0.18	0.10
Triglycerides (mg/dl)	0.28	0.008
Inflammatory Markers		
TNF α (pg/ml)	-0.21	0.08
CRP ($\mu\text{g/ml}$)	-0.15	0.22
Adiponectin ($\mu\text{g/ml}$)	-0.07	0.54
Carotid IMT (mm)	0.17	0.12

Table 3

A: Relationships between adjusted covariates and serum RBP4 levels (μg/ml) in multivariate regression modeling in all subjects including pre- and post-menopausal women (n = 88).

Parameter	Estimate (beta)	Standard Error	P value
FSH (IU/l)	0.04	0.02	0.03
Estradiol (pg/ml)	-0.02	0.02	0.21
SHBG (nmol/dl)	-0.02	0.02	0.20
Insulin AUC	0.0004	0.0003	0.23
Waist-to-hip ratio	6.18	9.94	0.54
Total Cholesterol (mg/dl)	0.02	0.02	0.28
Triglycerides (mg/dl)	0.007	0.02	0.69
TNFα (pg/ml)	-0.002	0.02	0.93

B: Relationships between adjusted covariates and serum RBP4 levels (μg/ml) in multivariate regression modeling in all subjects including pre- and post-menopausal women (n = 88).

Parameter	Estimate (beta)	Standard Error	P value
LH (IU/l)	0.12	0.06	0.04
Estradiol (pg/ml)	-0.03	0.02	0.10
SHBG (nmol/dl)	-0.02	0.02	0.21
Insulin AUC	0.0004	0.0003	0.22
Waist-to-hip ratio	4.16	9.89	0.68
Total Cholesterol (mg/dl)	0.02	0.02	0.22
Triglycerides (mg/dl)	0.008	0.02	0.65
TNFα (pg/ml)	0.003	0.02	0.90

Parameters with a $p < 0.1$ in univariate analysis were included in the multivariate regression model. The overall model attained an R^2 of 0.30 and was highly significant with a $p < 0.01$.

Parameters with a $p < 0.1$ in univariate analysis were included in the multivariate regression model. The overall model attained an R^2 of 0.29 and was highly significant with a $p = 0.01$.