

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

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*Running Title:* RBP4 in Polycystic Ovary Syndrome

Clinical trial registration: ISRCTN51367236

Received for publication 09 December 2007 and accepted in revised form 01 April 2008.

*Objectives:* Polycystic ovary syndrome (PCOS) is an insulin resistant (IR) state with IR an established therapeutic target, however measurement of IR remains challenging. We aimed to determine a) serum retinol-binding Protein 4 (RBP4) levels (purported to reflect IR) in PCOS women and controls, b) examine relationship of RBP4 to conventional IR markers and c) examine RBP4 changes with interventions modulating IR, in overweight PCOS women.

*Research Design and Methods:* 38 overweight women (BMI >27 kg/m<sup>2</sup>) with PCOS and 17 weight-matched controls were compared at baseline. PCOS women were then randomized to 6 months of higher dose oral contraceptive pill (OCP) (35mcg ethinyl estradiol/2mg cyproterone acetate) or metformin (1g bd). Outcome measures were IR [area under curve insulin] on oral glucose tolerance test, RBP4 and metabolic/inflammatory markers.

*Results:* Overweight women with PCOS were more IR than controls, yet RBP4 levels were not different in PCOS women vs. controls (35.4±4.3 vs. 28.9±3.1µg/ml, P=0.36). RBP4 correlated with cholesterol and triglycerides but not IR. Metformin improved IR by 35%, whilst the OCP worsened IR by 33%. However, RBP4 increased non-significantly in both groups (43.7±6.3 vs 42.6±5.5µg/ml, P=0.92).

*Conclusions:* Overweight women with PCOS were more IR than controls but this was not reflected by RBP4 levels. RBP4 correlated with lipid levels but not with IR markers. RBP4 levels did not change when IR was reduced by metformin or increased by the OCP. This data suggests that RBP4 is not a useful marker of IR in PCOS but may reflect other metabolic features of this condition.

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Polycystic ovary syndrome (PCOS) is the most common endocrinopathy affecting 7% of reproductive age women and up to 30% of obese women (1). Reproductive abnormalities are underpinned by insulin resistance (IR), which has a significant aetiological role in PCOS (1). Women with PCOS have increased IR compared to controls (matched for BMI and body-fat distribution) (1, 2) as well as increased metabolic syndrome (2) impaired glucose tolerance (IGT) and type 2 diabetes (DM2). However, IR is not included in the diagnostic criteria for PCOS. Challenges include inaccuracy of insulin assays, lack of clarity on optimal methods to assess IR and ill-defined cut-off values to define IR (2). A reproducible, accurate marker of IR, which 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 IR states, adipose tissue, (not skeletal muscle) GLUT4 is down-regulated (3). Recent convincing data in mice, suggests a strong causal link between RBP4 and IR. Adipose-specific GLUT4 knockout mice exhibit IR with increased adipose RBP4 (3). Over-expressing RBP4 in mice induces IR, whereas decreasing RBP4 reduces IR. Furthermore, RBP4 levels can be normalized by insulin-sensitizers and fenretinide, which reverse IR in obese rodents. Increased RBP4 impairs insulin signaling in muscle and increases gluconeogenesis in the mouse liver, suggesting RBP4 is involved in the pathogenesis of IR and is a marker of IR in mice.

In contrast, human data are equivocal with high adipocyte and plasma RBP4 levels, reported inconsistently in IR states, including obesity, IGT, DM2 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 which reduce IR, 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 IR state and IR *per se* is an important therapeutic target. Assessment of IR is likely to guide treatment with options including the OCP (which can increase IR) or metformin which reduces IR (2, 12). However, significant challenges remain in the measurement of IR (2). Although RBP4 shows promise as a marker of IR 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 RBP4 levels in overweight women with and without PCOS. We also examined the relationship between serum RBP4 levels and conventional IR markers, other metabolic factors and adiposity in women with PCOS. Finally, we examined the effect on serum RBP4 levels, of therapeutic interventions, which both increase and decrease IR in women with PCOS.

## RESEARCH DESIGN AND METHODS

**Subjects** This study comprises a subset of subjects from a larger pharmacologic intervention study (12). The subset reported here includes 38 PCOS women who were sequentially recruited, randomized to treatment group and completed the study intervention. Overweight women (BMI>27 kg/m<sup>2</sup>) with PCOS (n=38) and overweight controls (n=17) were recruited from community advertisements. PCOS diagnosis was based on perimenarchal onset of irregular cycles (<21 days or >35days) and clinical hyperandrogenism (hirsutism, acne) or biochemical hyperandrogenism (elevation of at least one circulating ovarian androgen) (1990 NIH criteria). Secondary causes of

amenorrhea and hyperandrogenism were excluded with clinical screening and early follicular 17-hydroxy progesterone levels. DM2 was excluded on oral glucose tolerance test (OGTT) (World Health Organization criteria). Pregnancy tests were negative prior to enrolment. The Southern Health Research Advisory and Ethics Committee approved the study and all participants gave written informed consent.

**Study design** At screening (3 months prior to baseline), standard diet and lifestyle advice was delivered (National Heart Foundation of Australia recommendations) and medications affecting IR including the oral contraceptive pill (OCP) were ceased. At baseline, PCOS women were randomized, based on computer generated random numbers, to either metformin 1g bd (dose titrated up over 4 weeks starting at 500mg bd) or higher dose OCP (35mcg ethinyl estradiol/2mg cyproterone acetate) in an open label study. The higher dose OCP is a commonly prescribed OCP in PCOS in Australia and Europe. Participants were reviewed by the same investigator at screening, baseline, 3 and 6 months after intervention. Data collection was completed by the research nurse, who was blinded to treatment allocation.

**Clinical and biochemical measurements** Subjects were weighed lightly clothed without shoes, BMI was calculated, [weight (kg) / height squared ( $m^2$ )], waist and hip circumferences were measured at the umbilicus and greater trochanter. The waist-hip ratio (WHR) was calculated as waist / hip circumference. Fasting blood samples were taken for endocrine and metabolic variables and an OGTT was measured 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, LDL, triglycerides, highly sensitive C reactive protein (hsCRP) (12) and RBP4. The free androgen index

(FAI) was calculated from  $FAI = (testosterone/SHBG) \times 100$ . A 120-minute 75-g OGTT was performed and further blood samples were taken for assessment of glucose and insulin at 30, 60, 120 and 180 minutes. Total insulin area under the curve (AUC insulin) during the OGTT was calculated geometrically using the trapezoidal rule. The homeostatic model assessment (HOMA) was used as a surrogate measure of insulin sensitivity and was calculated as  $fasting\ serum\ insulin\ (mU/L) \times fasting\ plasma\ glucose\ (mmol/L)/22.5$  as previously described (12). Due to erratic menstrual cycles, data was not collected at a specific cycle stage in PCOS women. Control data was collected during the follicular phase.

#### **RBP4**

Quantitative western blotting was performed based on the methods described by Graham et al (4, 11). Briefly, full-length recombinant RBP4 protein concentration (Cayman Chemical Company, MI, USA) was determined using the Bradford method (Bio-Rad, CA, USA). Standard solutions of 25, 50, 100 and 150 $\mu$ g/ml RBP4 were prepared in a standard buffer containing 0.1% BSA; 1% NP-40. Human sera and standards were then diluted 1:10 into a 1X LDS-PAGE sample buffer and heated for 5 minutes at 95°C. 15 $\mu$ l of diluted standards and samples in addition to molecular weight markers were loaded on 16% Tris-glycine pre-cast SDS-PAGE cassette gels (PAGEgel; CA, USA) and transferred to nitrocellulose membranes for immunoblotting. Nitrocellulose membranes were then blocked in solution containing 5% non-fat milk in Phosphate Buffered Saline and Tween-20 (TPBS). Blots were probed overnight with primary anti-body (anti-RBP4; Sapphire Biosciences, NSW, Australia) diluted 1:500 at 4°C. Following washing with TPBS, blots were rocked for 1hour in secondary anti-body (polyclonal goat anti-rabbit immunoglobulin HRP; DAKO cytometry, NSW, Australia) diluted 1:1000 at room temperature. Bands were detected by enzymatic chemiluminescence

(Millipore, MA, USA) and quantified using a luminescence imaging program (Multigauge, FujiFilm, Tokyo, Japan).

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

**Statistics** All data are presented as mean  $\pm$  SEM and log-transformed if not normally distributed. Results are presented for 55 subjects, 38 PCOS treated with metformin (n=19) or the OCP (n=19) and 17 controls, except for hsCRP (n=43) with 12 excluded with levels  $>10$  mg/L, potentially attributable to other inflammatory processes. Two-tailed statistical analysis was performed using SPSS for Windows 14.0 software (SPSS Inc, Chicago, USA) with statistical significance set at  $\alpha$  level of  $P \leq 0.05$ . Baseline data were assessed using one-way ANOVA with PCOS status as between subject factor. PCOS group was assessed using one-way ANOVA with intervention as between subject factor and comparisons between time points were assessed using repeated measures ANOVA with intervention as between subject factor. Relationships between variables were examined using bivariate (Pearson) correlations. Change in variable was defined as ratio of pre-treatment value and post-treatment value.

## RESULTS

All 17 controls and 38 PCOS women were screened then completed the 3 month run-in.

**Controls vs. PCOS women at baseline** Baseline clinical, anthropometric and endocrine characteristics are listed in table 1. The control and PCOS groups were similar in age, BMI and WHR. Testosterone, FAI, HDL and triglycerides were higher in the PCOS group (table 1). Two markers of IR, HOMA and fasting insulin, were higher in PCOS (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 indices of IR, hyperandrogenism or adiposity.

### **Intervention Study:**

There were no differences in baseline characteristics for PCOS subjects randomized to metformin (n=19) or 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 to 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 ( $P = 0.01$ ) and Triglycerides ( $P < 0.01$ ). HDL decreased with metformin and did not change with OCP. Triglycerides increased with the OCP, with no change with metformin. LDL decreased with the OCP, with no time by treatment effect. At completion, the OCP group demonstrated higher HDL ( $P < 0.01$ ). There was a time by treatment effect for hsCRP ( $P = 0.03$ ) with non-significant reduction with metformin and a non-significant increase with OCP.

**IR** 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 (Figure 1A) and the groups were significantly different post-treatment.

**RBP4** Despite decreased IR with metformin and increased IR with the OCP, RBP4 levels did not change in either group. There was no difference between groups in RBP4 following intervention (Figure 1B).

**Correlations with change in RBP4** Within the 2 groups, change in RBP4 did not correlate with change in IR, metabolic factors or indices of adiposity.

## CONCLUSIONS

Recent comprehensive studies in mice provide convincing evidence of a link

between IR and RBP4 (3). However, human data has been less consistent. In the current human PCOS study, a known IR state, we have demonstrated based on HOMA, that women with PCOS are more IR than overweight controls, but do not have different RBP4 levels. Furthermore, in PCOS, RBP4 levels did not correlate with other IR markers and did not change with interventions including metformin (which reduced IR) and the OCP (which increased IR). This data suggests that RBP4 is not a useful marker of IR in PCOS.

To date, human studies have failed to clarify the role of RBP4 as a marker of IR. RBP4 has been reported as high and correlated inversely with insulin sensitivity (on euglycaemic-hyperinsulinaemic clamp), in subjects with IGT and DM2 and non-obese relatives of subjects with diabetes (4). These populations mostly had abnormal glucose metabolism (AGM), rather than isolated IR and were predominately male. In one comparison of obese non-DM2 and DM2 subjects, the relationship between RBP4 and IR was not independent of BMI (4). Thus the isolated contribution of IR, cannot be determined. Recently, RBP4 correlations with IR have been inconsistent. Cho et al. (6) reported high plasma RBP4 levels in weight-matched humans with IGT and DM2, however RBP4 did not correlate with IR. Morbidly obese patients had high RBP4 levels compared to lean controls, but notably also had AGM with elevated fasting glucose (5). RBP4 was not elevated in obese post-menopausal women without DM2 (8) and did not correlate with IR.

Obese women with PCOS present with reproductive abnormalities, before the onset of AGM and have extrinsic obesity related IR, in addition to intrinsic PCOS related IR. They are more IR than weight-matched controls (2). Hence women with PCOS provide a useful model to investigate whether RBP4 reflects IR independent of AGM. Women with PCOS diagnosed based on NIH criteria demonstrate more severe IR (13, 14). Despite demonstrating a difference in IR (HOMA) between overweight PCOS

women and controls, RBP4 did not differ between groups. Similarly, Hahn et al. (15) did not detect a difference between lean PCOS and lean controls, in RBP4. However, in a study in 10 obese, IR women with PCOS (7), higher RBP4 levels were seen compared with weight-matched controls. Of note, the ELISA method was used rather than the Western blot. Tan et al. (7) noted PCOS women had a significantly higher mean fasting glucose (upper limit of normal range) than control women and that RBP4 correlated with glucose, but not HOMA or insulin levels, suggesting that RBP4 reflects AGM, not IR. This is consistent with the current study with no difference in RBP4 in a population with IR but without AGM. Although PCOS is an IR state, PCOS *per se* and IR does not appear to be related to high RBP4 levels.

We did not demonstrate a correlation between serum RBP4 levels and markers of IR in overweight women with PCOS at baseline confirming previous observational PCOS studies (7, 15). Other than Graham et al. (4) most studies in weight matched DM2 and obesity, have not demonstrated a relationship with RBP4 and IR (5, 6, 8, 10). We showed a correlation with RBP4 and triglycerides, which has been previously noted (4, 15). RBP4 correlates with adiposity markers or AGM relatively consistently across other studies, however we did not demonstrate these in relatively homogeneous overweight PCOS women with predominately normal glucose tolerance.

In the current study, despite demonstrating decreased IR after 6 months of metformin and increased IR with the OCP in overweight women with PCOS, we were unable to show any relationship between change in IR and RBP4. RBP4 has been shown to change with interventions which reduce IR in other IR populations although results are variable. RBP4 (ELISA) decreased with 13% weight loss post lap-banding, although there was no decrease in IR (5). Similarly, no relationship was seen between RBP4 and decreased IR

during weight reduction in obese women (10). Conversely, 5% weight loss, did not change RBP4, despite a decrease in IR (8). In 60 subjects, exercise training improved IR (euglycaemic clamp). A significant decrease in RBP4 was detected via post-hoc analysis in the most IR of subjects, in whom exercise improved IR markedly (4). RBP4 levels are normalized in mice treated with the insulin-sensitizer, rosiglitazone. Treatment of human IGT subjects with an insulin-sensitizer, pioglitazone resulted in a paradoxical increase in RBP4 expression in adipose tissue, despite a decrease in IR. Serum RBP4 was unchanged with pioglitazone and metformin, consistent with the current study (9).

FAI is preferable to testosterone as a marker of androgen excess in PCOS women. In the current study, FAI and testosterone decreased with the OCP, with no change with metformin, consistent with 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 dyslipidaemia of PCOS has both similarities with DM2 (elevated triglycerides) and differences (HDL is not low) as noted here and in a large study by Legro et al (19). In the current study, HDL decreased with metformin and was unchanged with the OCP, whilst triglycerides increased with the OCP. Other studies have had similar findings (12, 17), yet a Cochrane review in PCOS (16) found no changes in HDL with metformin. This contrasts with DM2, where HDL increases with metformin (20). These observations 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 IR. Only studies using hyperinsulinaemic-euglycaemic clamps, found a correlation with RBP4(4). Western blotting yields RBP4 concentrations with a greater dynamic range than the ELISA (11). The ELISA assay may underestimate the differences in RBP4 between insulin-sensitive and IR subjects. EIA has been shown to undervalue RBP4 concentrations possibly due to assay saturation in IR. Some commercial ELISA assays 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 IR 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 IR, 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 IR, adipose-specific GLUT4 knockout mice (3). Work with remaining identified proteins may elucidate the relationship between the downregulation of adipose-specific GLUT4 in humans and IR.

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

Whilst women with PCOS are more IR than overweight controls, this was not reflected by RBP4 levels. RBP4 did not

correlate with other markers of IR in overweight women with and without PCOS. In addition, RBP4 did not change with changes in IR from therapeutic interventions including metformin (reduced IR) or the OCP (increased IR). RBP4 did correlate with lipid levels, suggesting that RBP4 is not a useful marker of IR, but may reflect metabolic abnormalities in PCOS.

#### **ACKNOWLEDGEMENTS**

Pathology was completed at Southern Health laboratories.

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. Professor Teede is an NH&MRC Fellow, Dr Hutchison is an NH&MRC PhD Scholar.

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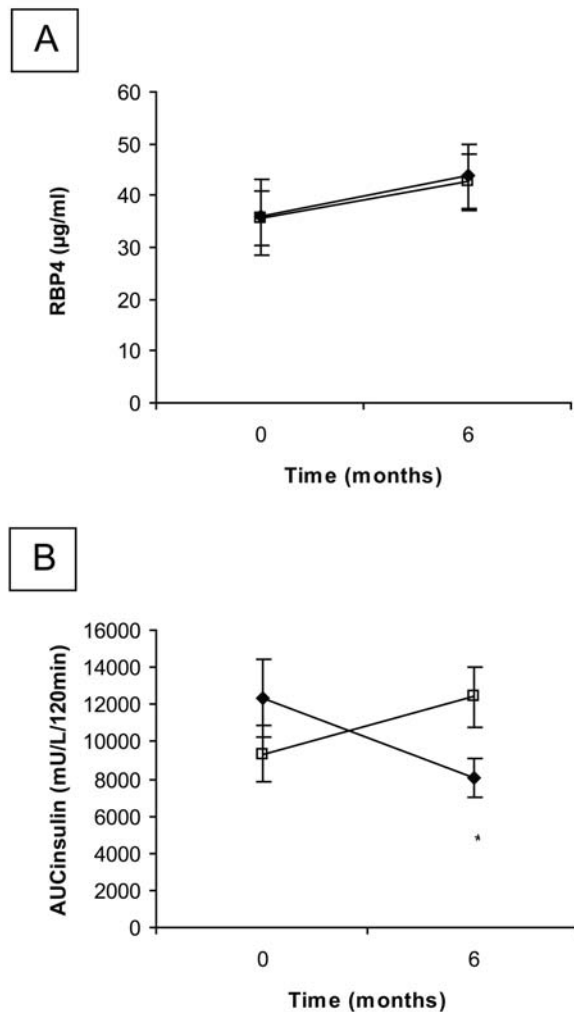


Figure 1: Changes in Retinol-binding protein 4 (A) and area under the curve insulin on oral glucose tolerance test (B) following metformin (black diamonds) or oral contraceptive pill (white squares) for 6 months.

Data is presented as mean  $\pm$  SEM. AUC: Area under the curve; Min: minute; OCP: oral contraceptive pill; RBP4: retinol-binding protein. 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 decrease in AUC insulin for metformin ( $P = 0.03$ ) and increase for OCP ( $P < 0.01$ )

Table 1: Characteristics at baseline of controls vs. PCOS

Baseline Characteristic	Controls	PCOS	P value
	(n = 17)	(n = 38)	
Age (years)	33.2 ±1.9	34.1 ±1.2	0.68
BMI (kg/m <sup>2</sup> )	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-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
Free Androgen Index	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 (mmol/L)	1 ±0	1.3 ±0.1	<0.01
LDL (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 is presented as mean ± SEM

hsCRP: highly sensitive C-reactive protein; SHBG: Sex hormone binding globulin; HOMA: homeostasis model assessment; RBP4: Retinol-binding protein 4.

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

Characteristic	Metformin n= 19		OCP n=19		P value for Change over study Metformin vs OCP
	Pre	Post	Pre	Post	
BMI (kg/m <sup>2</sup> )	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-Hip ratio	0.9 ±0	0.9 ±0	0.9 ±0	0.9 ±0	0.65
CRP (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
Free Androgen Index	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 (mmol/L)	1.2 ±0.1	1.1 ±0.1*	1.4 ±0.1	1.4 ±0.1†	0.01
LDL (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/120min)	12333.9 ±2046.4	8054.7 ±1090.6*	9339.8 ±1531.5	12413.5 ±1616.7*†	<0.01
HOMA	4.7 ±1.1	4.2 ±1.7	4.2 ±0.9	4 ±0.6	0.1
RBP4 (ug/L)	35.9 ±7.4	43.7 ±6.3	35.7 ±5.3	42.6 ±5.5	0.92

Data is presented as mean ± SEM

hsCRP: Highly sensitive C-reactive protein; SHBG: Sex hormone binding globulin; AUC insulin: Area under curve insulin; HOMA: homeostasis model assessment; RBP4: Retinol-binding protein 4

\* 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

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.