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**Endometria from Obese  
PCOS Women with  
Hyperinsulinemia Exhibit  
Altered Adiponectin  
Signaling**

DOI 10.1055/s-0035-1555806  
Horm Metab Res

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levels of APPL1 are paradoxically increased in skeletal muscle of type 2 diabetic and obese subjects, compared to lean controls; moreover, its expression is reduced in obese subjects with weight loss. Also, in the present work we did not find the upregulated expression of APPL1 that was expected because of the high levels of AdipoR1 in the obese group, regulatory mechanism identified in skeletal muscle of the rat model [50]. These data suggest that human endometrium of obese and obese-PCOS group presents an alteration of the signaling pathway beyond adiponectin receptors. These alterations could increase insulin resistance and affect the energy homeostasis in endometrial cells. In conclusion, the data of the present investigation suggest that the hyperinsulinism and hyperandrogenism conditions besides obesity present in women with PCOS could have an impact in endometrial function, provoking a disturbance in the adiponectin signaling pathway, which is corroborated in the in vitro model, and this could affect endometrial function and potentially the implantation process.

## Acknowledgements

The authors thank the laboratory team especially Rodrigo Carvajal (MD, PhD) for clinical evaluation of patients and Fernando Gabler (MD) for histological evaluation of endometrial samples. We are also grateful to the women who donated tissue. This study was supported by grants # 1130053 from the Fondo Nacional de Desarrollo Científico y Tecnológico, Chile (to MV), # 24121256 (to CR) and # 21120541 (to LO) from Comisión Nacional de Investigación Científica y Tecnológica, Chile.

## Conflict of Interest

The authors declare no conflict of interest.

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was significantly diminished with the combined effect of insulin plus testosterone (45%,  $p < 0.01$ ), and no changes were observed in the presence of insulin or testosterone alone (33% diminished,  $p > 0.05$ ). Again, with the combined action of testosterone and insulin, AdipoR1 and AdipoR2 protein levels were diminished in St-T1b cell line cultures (45 and 46%, respectively vs. basal), although no significant effect was obtained with testosterone or insulin alone; the same results were obtained for APPL1. Therefore, these *in vitro* results indicate that the action of adiponectin could be disturbed by high concentrations of testosterone and insulin as in the PCOS situation, and are in agreement to those obtained in endometrial tissues, specifically, in the endometria from obese-PCOS women. Finally, testosterone or insulin alone did not elicit an important cell response as the combined hormonal effect.

## Discussion

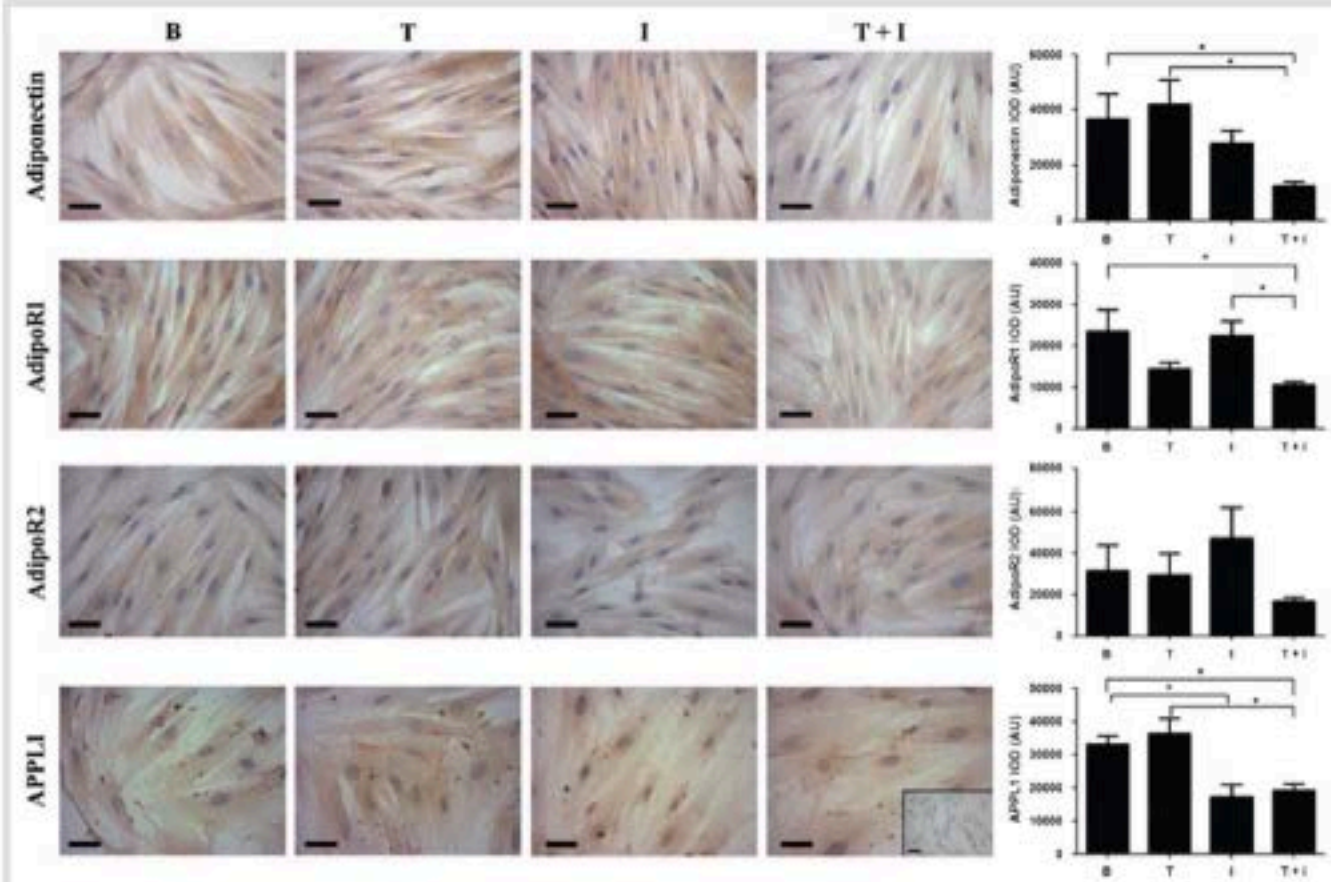
Polycystic Ovarian Syndrome (PCOS) is a highly prevalent disorder during the female reproductive age where hyperandrogenism and insulin resistance may coexist. Furthermore, a high percentage of women bearing PCOS are obese, and obesity is linked to insulin resistance at least in a subgroup of patients, where insulin resistance is worsened by excessive adipose mass. Recently, it has been described that the adipose tissue generates several polypeptides (adipocytokines) that may control food intake or regulate insulin sensitivity [28]. Regarding adipokines, different studies have demonstrated the relationship between adiponectin and female reproduction [29,30]. In the same way, in humans a relationship between obesity, PCOS, and low levels of plasma adiponectin has been described [31–33]. In addition, diminished plasma levels of adiponectin are observed in pre-eclampsia and in lean, obese, and pregnant women with PCOS [34,35].

Besides the ovarian dysfunction in PCOS, the appropriate function of the endometrium is also affected in these patients as evidenced in several investigations [8–12,36,37], although no reports are available concerning the adiponectin pathway molecules neither in human endometrium nor in the PCOS condition. One report has established that the receptors for adiponectin, AdipoR1 and AdipoR2, are expressed in the human endometrial cell compartments, epithelia and stroma, throughout the menstrual cycle, particularly during the secretory phase [16]. In addition, evidences show that adiponectin can be considered as an insulin sensitizer in many tissues where a relationship has been described between the insulin and adiponectin signaling pathways [14]. Recently, our group has reported alterations in the levels of molecules involved in the insulin pathway in endometria from PCOS patients, with different pattern of Akt phosphorylation, abnormal expression of IRS-1 and GLUT4 [8,12,36]. Potentially, these results could relate the adiponectin system and the insulin pathway in endometria.

As mentioned, in the present investigation it was found that serum adiponectin levels were lower in obese PCOS patients compared to lean and obese women without PCOS, in addition to altered parameters of insulin resistance (HOMA and ISI Composite) and higher FAI values in the obese PCOS group. These results are in agreement with previous reports, which established that BMI, IR, and FAI make a highly significant contribution to adiponectin levels in PCOS [38]. The link between FAI, IR and adiponectin levels may exist for several reasons. Prospective

studies have shown a relationship between IR and decreased adiponectin levels with weight gain and increased waist circumference [39]. Furthermore, since androgens promote central adiposity and visceral adipose deposition, differences in adipose tissue distribution may influence the secretion of the different adipocytokines and pro-inflammatory proteins, such as TNF- $\alpha$  and IL-6, which are upregulated in PCOS patients [40]; these cytokines may downregulate the expression of adiponectin. Additionally, in the present study the expression of adiponectin and its receptors (AdipoR1 and AdipoR2) and the adaptor protein APPL1 was evaluated in human endometrium from lean, obese and obese hyperinsulinemic PCOS women for the first time. The analysis of adiponectin content assessed by Western blot shows lower protein levels in obese-PCOS endometria compared with the other 2 groups. This is consistent with our observation in the *in vitro* model (T-HESC and St-T1b cell lines), where supraphysiological concentration of testosterone and insulin, provokes a diminution in adiponectin protein expression. The mechanism by which these hormones can disturb adiponectin levels in the human endometrium is presently unknown, although in the PCOS condition the proinflammatory state besides obesity and insulin resistance may induce endoplasmic reticulum (ER) stress [41].

Furthermore, the analysis of AdipoR1 illustrates the protein expression in human endometrium, being higher in the obese group compared to the lean and obese-PCOS groups. The molecular mechanism that involves the increase of AdipoR1 in the endometrium of the obese group is unclear. Interestingly, this response is not observed in endometria from obese-PCOS patients probably due to their conditions of hyperinsulinism and hyperandrogenism. A number of studies in mice and human have shown a positive correlation between hyperinsulinemia and downregulation of AdipoR1/R2 in different cellular types like skeletal muscle, liver, and adipose tissue [42,43]. Nevertheless, the relationship between hyperandrogenism/hyperinsulinism and the expression of AdipoR1/AdipoR2 in human endometrium has not been addressed before. Importantly, in our *in vitro* model, the response of endometrial cells to the action of insulin plus testosterone is diminution of adiponectin, AdipoR1 and AdipoR2 protein levels, suggesting that in the human endometrium, adiponectin and its receptors can be hormonally regulated. Moreover, the disturbance in the expression of the adiponectin system and its receptors in this tissue could affect the endometrial changes for implantation. As previously described, adiponectin and its receptors are increased during the implantation period, that is during the midluteal phase; and it is relevant that AdipoR1 and AdipoR2 mRNA and protein, are both reduced in patients with implantation failure [44,45]. Even more, reports have indicated that adiponectin and its receptors induce activation of AMPK in epithelial and stromal cells, suggesting that adiponectin may regulate energy balance in these cellular types involved in the process of endometrial receptivity and implantation [12,16]. Therefore, in the present study the content of the adaptor protein APPL1 was evaluated in endometrial tissue of lean, obese and obese-PCOS women. The results show, for the first time, a decrease in APPL1 levels in human endometrium from obese and obese-PCOS patients. These results are in agreement with previous investigations that report a decrease of APPL1 levels in conditions of hyperglycemia and obesity [46] and with those from rodent models with diabetes and obesity [47,48]. However, our observation is in disagreement with the work of Holmes et al. [49], which indicate that the



**Fig. 3** Immunocytochemical evaluation of adiponectin, AdipoR1, AdipoR2, and APPL1 in the endometrial stromal cell line T-HESC. Left panels: Micrographs showing positive staining of adiponectin, AdipoR1, AdipoR2, and APPL1 in T-HESC cultures under testosterone (T), insulin (I), or testosterone plus insulin (T+I) treatment. Insert corresponds to negative control (first antibody is omitted). Scale bar = 50  $\mu$ m. Right panel: Semi-quantitative analysis of positive staining by the IOD tool in cell line T-HESC cultures in the absence (Basal) or presence of T, I, or T+I (100nM each). Results are expressed as Means  $\pm$  SEM of AU; n=3 in duplicate, \* p-value <0.05 vs. basal. (Color figure available online only).

obese-PCOS exhibit lower protein levels of adiponectin, AdipoR1, and APPL1 relative to endometria obtained from obese and/or lean patients.

#### Messenger RNA levels of AdipoR1, AdipoR2, and APPL1 in endometrial tissues by quantitative PCR

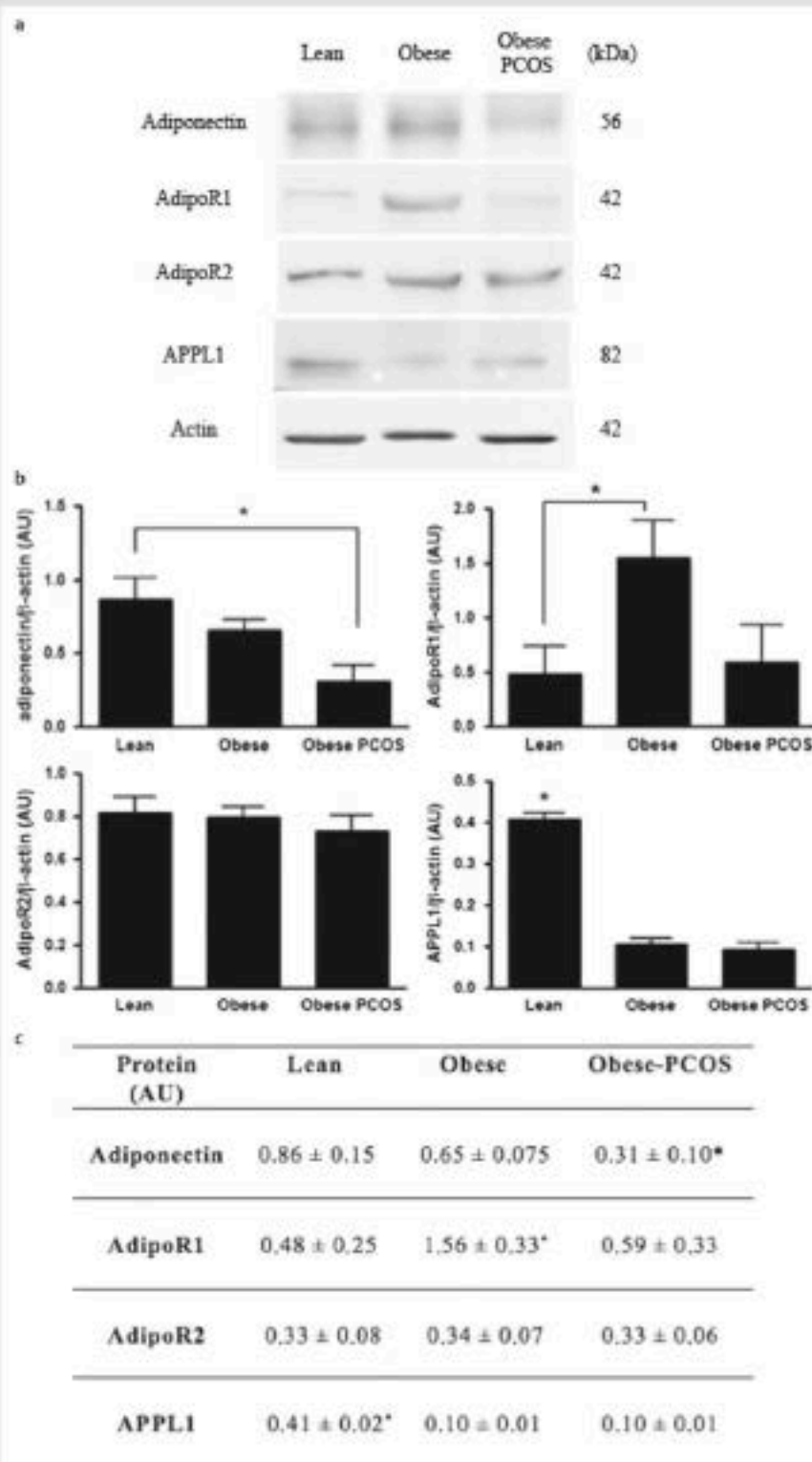
To assess whether the obesity and/or the PCOS condition could affect the transcriptional process for adiponectin receptors and APPL1 in human endometria, real-time PCR analysis was performed. The results for AdipoR1 mRNA show an increase, although not significantly different, in endometria from obese women compared to the lean group, and to the obese-PCOS endometria (67 and 52%, respectively), coincident with the high protein level for AdipoR1 found in those endometria. The AdipoR2 mRNA level was similar between the 3 studied groups (Lean:  $0.95 \pm 0.1$ ; Obese:  $1.16 \pm 0.6$ ; Obese-PCOS:  $1.7 \pm 0.9$  AU, Media  $\pm$  SEM). When APPL1 mRNA was assayed no differences were found between the 3 studied groups of endometria (Lean:  $0.04 \pm 0.009$ ; Obese:  $0.04 \pm 0.003$ ; Obese-PCOS:  $0.078 \pm 0.003$  AU, Media  $\pm$  SEM).

#### Adiponectin, AdipoR1, AdipoR2, and APPL1 protein levels in endometrial cell lines under hormone stimuli

To address the question of whether high blood levels of androgens and/or insulin found in the group of obese-PCOS could alter the protein content of adiponectin, its receptors and APPL1, an in

vitro cell model was used. Stroma endometrial cell lines (T-HESC and St-T1b) were cultured in the presence or not of supraphysiological concentrations of testosterone and/or insulin, mimicking partially the PCOS condition, where adiponectin, AdipoR1, AdipoR2, and APPL1 levels were analyzed by immunocytochemistry using the IOD tool ( $\odot$  Fig. 3). The results show that the studied proteins are detected in the 2 endometrial cell lines under the different culture conditions ( $\odot$  Fig. 3a, b for T-HESC). The semi-quantitative analysis with the IOD tool shows that the addition of testosterone plus insulin to T-HESC cultures elicits a significant decrease in the protein levels of adiponectin compared to basal (66%,  $p < 0.05$ ), while no significant differences were obtained after the addition of testosterone or insulin alone. When evaluating AdipoR1 protein levels under the combined action of testosterone and insulin, a significant decrease was found in T-HESC ( $p < 0.01$ ), and a similar response was obtained for AdipoR2 protein levels. The addition of testosterone to cultures elicited a nonsignificant decrease in AdipoR1 protein content (39%), whereas AdipoR2 levels were similar to basal. For APPL1, a significant decrease of protein levels was obtained when T-HESC were incubated in the presence of testosterone plus insulin (42%,  $p < 0.05$ ); also, the addition of insulin alone provoked a similar decrease in APPL1 protein content (48%,  $p < 0.05$ ).

On the other hand, St-T1b cell line responded similarly than T-HESC to hormone stimuli. In fact, adiponectin protein content



**Fig. 2** Western blot analysis of protein levels of Adiponectin, AdipoR1, AdipoR2, and APPL1 in endometria obtained from Lean, Obese, and Obese-PCOS;  $n = 9$  for each group. **a** Representative gel of equal amounts of protein (50  $\mu$ g) loaded in each lane. **b** Band intensities were quantified by scanning densitometry and normalized to intensities observed for beta-actin as internal control, results expressed as Means  $\pm$  SEM AU. \*  $p$ -value  $< 0.05$ . **c** Table showing Means  $\pm$  SEM AU of each protein in the 3 studied groups. \*  $p$ -value  $< 0.05$  Lean vs. Obese-PCOS for Adiponectin; Obese vs. Lean for AdipoR1; Lean vs. other groups for APPL1.

#### Protein levels of Adiponectin, AdipoR1, AdipoR2, and APPL1 in human endometria by Western blot

To further assess the protein content of adiponectin, AdipoR1, AdipoR2, and APPL1, Western blot analysis were performed by comparing protein content between lean, obese, and obese-PCOS endometria ( $\square$  Fig. 2a). A reduction of adiponectin protein levels in endometria from obese-PCOS group was determined compared with the lean group (64%,  $p < 0.05$ ). When analyzing the protein content of type 1 receptor of adiponectin in the 3

different endometria, a significant increase was determined in tissue from the obese group relative to the lean group (69%,  $p < 0.05$ ) and to obese-PCOS (62%) ( $\square$  Fig. 2b, c), whereas all studied endometria exhibited similar protein levels for AdipoR2. Moreover, a significant higher protein content of APPL1 was obtained in endometria from lean women compared to those from obese and obese-PCOS groups (76%,  $p < 0.05$ ), while no differences were observed in protein levels between the obese groups ( $\square$  Fig. 2b, c). Therefore, the endometria obtained from

**Table 1** Chemical and endocrine characteristics of studied groups.

Parameters	Lean	Obese	Obese-PCOS
Age (years)	26.6 ± 5.6	27.9 ± 5.2	25.9 ± 2.4
BMI (kg/m <sup>2</sup> )	22.4 ± 2.1	34.7 ± 4.8 <sup>§</sup>	32.6 ± 3.4 *
Estradiol (pmol/l)	168.7 ± 77	151.3 ± 73	143.6 ± 40
Testosterone (ng/dl)	36.6 ± 10.7	22.6 ± 8.4	49.4 ± 9.2 *
Androstenedione (ng/ml)	4.9 ± 1.3	3.5 ± 1.6	5.3 ± 1.4
SHBG (nmol/l)	68.9 ± 0.8	43.0 ± 24	25.7 ± 11 *
Free androgen index (FAI)	1.9 ± 0.6	2.3 ± 0.7	6.7 ± 1.9 *
Adiponectin (ng/ml)	85.0 ± 24.7	73.5 ± 31.7	47.7 ± 7.8 *

Lean, Obese, and hyperinsulinemic Obese-PCOS. Values are means ± SEM; n = 9 for each group

\* p-value < 0.05 between obese-PCOS and lean groups; § p-value < 0.05 between Obese and Lean groups

BMI: Body mass index; SHBG: Sex hormone-binding globulin

**Table 2** Metabolic parameters of studied groups.

Parameters	Lean	Obese	Obese-PCOS
Fasting glucose (mg/dl)	96.1 ± 12.4	89.1 ± 7.2	92.9 ± 11.7
Glucose 120 min (mg/dl)	96.1 ± 15.7	124.3 ± 26.5	122 ± 21.6
Fasting insulin (μU/ml)	7.8 ± 3.5	10.5 ± 3.3	17.1 ± 6.0
Insulin 120 (μU/ml)	45.6 ± 15.4	59.4 ± 20.6	150.5 ± 86.6 *
HOMA IR	1.49 ± 1.1	2.3 ± 0.86	4.5 ± 0.9 *
ISI composite	8.1 ± 4.7	4.6 ± 2.3	2.09 ± 1.1 *

Lean, Obese, and hyperinsulinemic Obese-PCOS. Values are Means ± SEM; n = 9 for each group

HOMA-IR: Homeostasis model assessment insulin resistance = Insulinemia

(μU/ml) × glycemia (mg/dl) / 405; ISI Composite, Insulin Sensitive

Index = 10,000 / (G<sub>0</sub> × I<sub>0</sub> × G<sub>120</sub> × I<sub>120</sub>), where G<sub>0</sub>: basal glycemia; I<sub>0</sub>: basal insulinemia;

G<sub>120</sub>: glycemia post OGTT; I<sub>120</sub>: insulinemia post OGTT

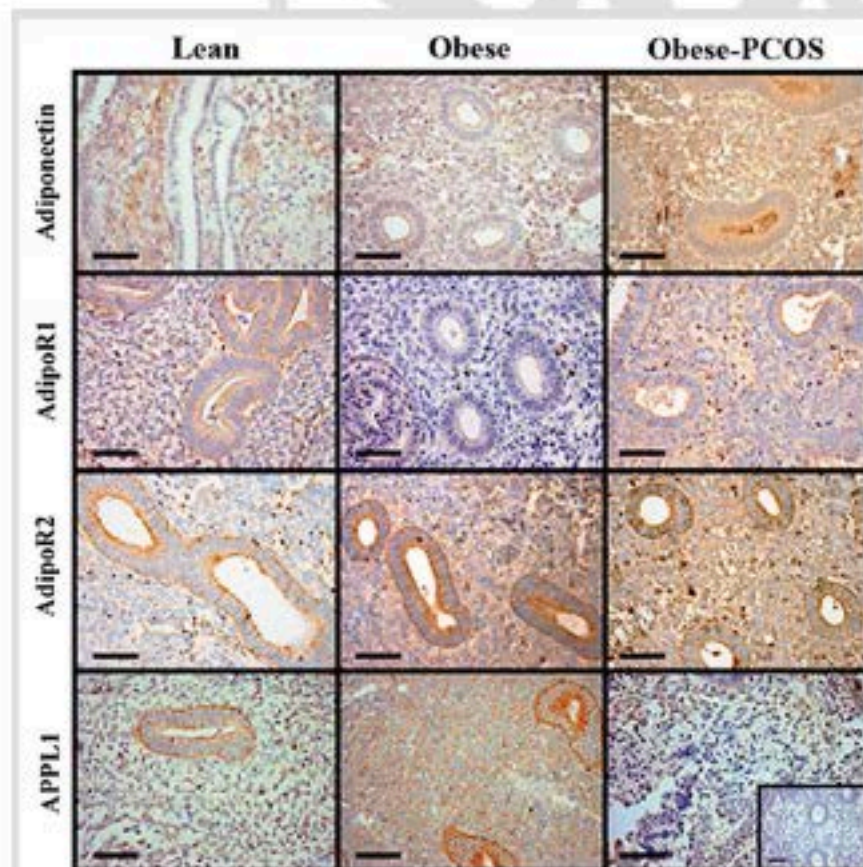
\* p-value < 0.05 between Obese-PCOS and Obese and Lean groups

diagnosed as women with hyperinsulinemia. The ISI composite value is lower in the obese-PCOS group compared to the other 2 studied groups, corroborating the hyperinsulinemic status in that group of patients (○ Table 2). Similarly, the adiponectin serum levels were lower in the obese-PCOS group compared to lean women (p < 0.05) and to the obese group. According to the experimental design, the group of obese-PCOS women participants in the present investigation, besides obesity presents hyperandrogenism and hyperinsulinism, characteristics not present in the other 2 studied groups.

#### Detection of adiponectin, AdipoR1, AdipoR2, and APPL1 in human endometria by immunohistochemistry

The adiponectin, AdipoR1, AdipoR2, and APPL1 proteins were examined by immunohistochemistry in the different compartments of the human endometria obtained from lean, obese, and obese-PCOS women (○ Fig. 1).

Positive staining for adiponectin was predominantly detected in the cytoplasm of the endometrial epithelial and stromal compartments in all studied groups. Additionally, AdipoR1 and AdipoR2 proteins were also found in the cytoplasm of epithelial and stromal compartments of all studied endometria. Lower staining for AdipoR1 was observed in the obese group compared with the lean group, similarly to that obtained in the obese-PCOS group. On the other hand, it was found that APPL1 positive staining in the endometria from obese-PCOS group was greatly diminished in both cell compartments, whereas an increase of the positive staining of APPL1 was detected in the endometria from lean group of patients compared with the obese-PCOS group.



**Fig. 1** Immunohistochemical detection of adiponectin, AdipoR1, AdipoR2, and APPL1 proteins in paraffin wax sections of proliferative endometria obtained from 27 samples from lean (left column), obese (central column), and obese-PCOS (right column) patients; n = 9 for each group. Positive staining was detected in epithelial and stromal cells of all studied endometria for all antigens. As a negative control (Insert), the primary antibody was omitted. Scale bar represents 50 μm. (Color figure available online only).

using random primers in a total volume of 20  $\mu$ l. PCR amplification of studied mRNA was assessed by using gene-specific primers: for APPL1: Sense 5'-TTA GCT GCC CGG GCC ATC CAT A-3'/Antisense 5'-ATC TTT TCC CCC TCA TTG TTT G-3'; for AdipoR1: Sense 5'-AAA CTG GCA ACA TCT GGA CC-3'/Antisense 5'-GCT GTG GGG AGC AGT AGA AG-3' and AdipoR2: Sense 5'-ACA GGC AAC ATT TGG ACA CA-3'/Antisense 5'-CCA AGG AAC AAA ACT TCC CA-3'. GAPDH gene was used as an internal control: Sense 5'-GAG TCA ACG GAT TTG GTC GT-3'/Antisense 5'-ATC CAC AGT CTT CTG GGT G-3'. Real-time PCR was performed on the Step One PCR System (Applied Biosystems). The reaction mixture (20  $\mu$ l) contained 1  $\mu$ l of diluted cDNA sample (equivalent to 20 ng of initial total RNA), 0.5  $\mu$ l of each primer (10  $\mu$ M), and 10  $\mu$ l of SYBR® Green QPCR Master Mix 2X. For negative control reaction, no template was added to the reaction mixture. Values were expressed as relative copies of each transcript and results are shown as a ratio of GAPDH internal control. The mean of these values were obtained per each studied group and expressed as Means  $\pm$  SEM.

### Immunohistochemistry and immunocytochemistry

Paraffin sections of human endometrial tissue were deparaffinized in xylene and gradually hydrated through graded alcohols. The slides were incubated in 10 mM citrate buffer (pH 6.0) at 95 °C for 30 min, incubating the samples in 3% hydrogen peroxide for 15 min preventing endogenous peroxidase activity. Non-specific antibody binding was inhibited by incubating samples with the blocking solution (Histostain-SP, Invitrogen, MD, USA) for 10 min. Then, samples were incubated with corresponding primary antibodies: adiponectin (monoclonal, Novus Biological, 1/100), AdipoR1 (polyclonal, Alpha Diagnostic, 1/500), AdipoR2 (polyclonal, Alpha Diagnostic, 1/1 000), and APPL1 (monoclonal, Cell Signaling, 1/200) overnight at 4 °C. The internal control was carried out on adjacent sections incubated without the primary antibody. A biotinylated HRP-secondary antibody was used for the detection of immune signal. Chromogenic revealed was developed by the streptavidin-peroxidase system and 3,3'-diaminobenzidine was used as substrate; counterstaining was performed with hematoxylin (Dako, CA, USA). The slides were observed on an Olympus optical microscope, and a histological description for each sample was performed.

For immunocytochemical assays, T-HESC and St-T1b cells were fixed with a solution containing 4% paraformaldehyde in PBS for 15 min at room temperature. The endogenous peroxidase activity was inhibited by incubation in 3% H<sub>2</sub>O<sub>2</sub> and nonspecific binding was blocked with PBS-BSA 2%. Cells were incubated with the primary antibodies (Adiponectin, AdipoR1, AdipoR2, and APPL1 with the above mentioned dilutions) overnight at 4 °C, rinsed with PBS 1X solution 3 times and incubated with a HRP-labeled secondary antibody to Rabbit IgG (KPL, MD, USA; 1/300) for 2 h at 37 °C. Chromogenic revealed and counterstaining were performed as for the immunohistochemistry assays. Slide analysis was performed by the measurement of positive pixel intensity in at least 1 000 cells with the use of the semi-quantitative analysis tool-integrated optical density (IOD) in the Image-Pro Plus 6.2 program. The data are presented as IOD Arbitrary Units (AU). The mean of these values were obtained per sample and studied condition and expressed as Means  $\pm$  SEM.

### Western blotting

Total proteins (50  $\mu$ g obtained from endometrial tissue) were denatured and fractionated using 8% SDS-PAGE gels. The protein

content was transferred to a nitrocellulose membrane and protein transference was confirmed by Ponceau S staining. The membranes were blocked for 1 h in TTBS (20 mM Tris, pH 7.5; 137 mM NaCl; 0.1% Tween 20) containing 5% nonfat dry milk. Membranes were washed twice for 5 min with TTBS and incubated overnight at 4 °C with antibodies against adiponectin (1:200), AdipoR1 (1:200), AdipoR2 (1:100), and APPL1 (1:200). Beta-actin antibody (Sigma-Aldrich; 1:20 000) was incubated for 1 h at room temperature. Blots were washed 3 times for 5 min each with TTBS, followed by incubation for 1 h at room temperature with anti-rabbit IgG peroxidase-linked (1:10 000), while rocking. After antibody incubation, the membranes were washed 3 times for 5 min each with TTBS; the bound antibodies were detected with an enhanced chemiluminescence substrate, Western Lightning Plus-ECL (PerkinElmer). Band intensities were quantified by scanning densitometry utilizing the UN-SCAN-IT software, Automated Digitizing System, version 5.1. The band intensity data are presented as arbitrary units (AU) and the results are expressed as a ratio of the beta-actin internal control. The mean of these values were obtained per each studied group and expressed as Means  $\pm$  SEM.

### Enzyme linked immunosorbent assay (ELISA)

Serum samples from the 3 studied groups were used for detection of adiponectin levels by Quantikine® ELISA Human Total Adiponectin/Acrp30 (R & D Systems, Catalogue #DRP300). Protein was detected by a colorimetric reaction using an ELISA reader (Biotek EL800) and absorbance measurements at 450 nm were performed using Gen5 1.06 program.

### Statistical analysis

The number of total subjects was 27. The calculated number per group was 9 assuming  $\alpha=0.05$  and  $\beta=0.2$ , according to our previous studies [8,9]. The distribution of the data was analyzed by Kolmogorov-Smirnov test. The ex vivo and in vitro results were analyzed by Mann-Whitney or Student's t-test, respectively. For multiple comparisons, ANOVA or Kruskal-Wallis statistical tests were used according to the nature of the results. All p-values < 0.05 were considered significant. Statistical tests were performed using Stata 9 and Graph Pad Prism 5.0.

## Results

### Clinical and endocrine characteristics of studied patients

The clinical and endocrine characteristics of the 3 groups of women (lean, obese and obese-PCOS) are summarized in Table 1. The age of the studied groups was similar, whereas, the BMI was significantly higher in the obese and obese-PCOS groups compared to the lean group ( $p<0.05$ ), according to the experimental design. In addition, the high ovarian androgen production besides the decreased SHBG blood level leads to a significantly higher FAI in PCOS obese women ( $p<0.05$ ). Progesterone levels (<3 nmol/l), in addition to the morphological evaluation by the pathologist, confirmed that all endometrial biopsies were obtained during the proliferative phase. Moreover, a histological evaluation of endometria from the 3 studied groups shows no differences between them (data not shown), as we have previously reported [8]. Besides, obese-PCOS women presented hyperinsulinemia and insulin levels 120 min post-load of glucose over the normal value (Table 2); therefore, they were

the PCOS condition, which could be related to alterations in their implantation capacity.

Moreover, the prevalence of obesity in women with PCOS has increased to around 70% as recently shown [13]. The obesity condition leads to a change in adipocyte function, altering their production of adipokines such as adiponectin, an obesity down-regulated insulin-sensitizing and antidiabetogenic adipokine [14,15]. Regarding the endometrium, adiponectin and its receptors, AdipoR1 and AdipoR2, have been found in this tissue throughout the menstrual cycle [16,17]. However, little information is available related to adiponectin and its effect in this tissue. Adiponectin signal transduction pathway includes APPL1 (phospho-tyrosine interaction, PH domain and leucine zipper containing 1), an adaptor molecule that transmits the signal from adiponectin receptors to regulate cell metabolism and insulin sensitivity, including glucose uptake and GLUT4 translocation-related proteins [18,19]. Nevertheless, it is unknown whether the expression of proteins from the adiponectin signaling pathway is altered in endometria from PCOS women. Consequently, based on evidences showing that adiponectin has biological implications for female fertility [20], in the following investigation, we have evaluated serum adiponectin concentration and the mRNA and protein levels of adiponectin, its receptors and APPL1 in endometria obtained from obese PCOS women, characterized by biochemical hyperandrogenemia and hyperinsulinemia. Additionally, protein levels of adiponectin, its receptors and APPL1 were assessed in an *in vitro* model of endometrial cells under steroid stimulation resembling the PCOS condition.

## Subjects and Methods

### Subjects

Human endometria were obtained with a Pipelle suction curette from the corpus of the uteri of lean, obese, and hyperinsulinemic obese women with PCOS. Therefore, 27 women were recruited and classified in 3 groups: lean ( $n=9$ , Body Mass Index, BMI <25); obese ( $n=9$ , BMI >30), and obese-PCOS ( $n=9$ , BMI >30). PCOS was diagnosed by the Androgen Excess and PCOS Society criteria [3]. The group obese-PCOS was the only one bearing PCOS with hyperinsulinemia besides hyperandrogenemia. Lean and obese groups could present oligoovulation or anovulation or polycystic ovaries assessed by ultrasound, although without hyperandrogenism and hyperinsulinism. None of the women participants in the present investigation had received any hormonal or other treatment in the last 3 months prior to recruitment. Endometria were obtained during the proliferative phase of the menstrual cycle, and the endometrial dating was done according to Noyes criteria [21] by an experienced pathologist. Also, blood samples were obtained and the circulating serum levels of estradiol, progesterone, testosterone, and sex hormone-binding globulin (SHBG) (by chemiluminescence) and adiponectin (by ELISA commercial kit) were assessed. Biochemical hyperandrogenism was determined by the Free Androgen Index (FAI) that considers total testosterone and SHBG blood levels (normal value <4.5). Glucose and insulin levels were evaluated by an oral glucose tolerance test (OGTT) with 75 g load of glucose (fasting glucose <110 mg/dl; glucose 120 min <140 mg/dl). To determine the hyperinsulinemic condition, serum glucose and insulin levels were measured at 120 min post load of glucose. The diagnosis of hyperinsulinemia was determined when 120-min insulin levels were twice the standard deviation of insulin

concentration over the mean of the control group, as in previous studies [8,12,22]. Additionally, Homeostasis Model Assessment (HOMA) index (normal values <3.0) and the Insulin Sensitive Index (ISI) Composite (normal values >3.0) were calculated for all patients [23,24]. The exclusion criteria were women who presented hyperprolactinemia (prolactin >35 ng/ml), hypothyroidism (TSH >5 mIU/ml), androgen-secreting tumors (total testosterone >2 ng/ml; dehydroepiandrosterone sulfate >3 600 µg/ml), Cushing's syndrome (urine cortisol concentration >150 µg/24 h and fasting plasma concentration of cortisol between 5 and 25 µg/dl), congenital adrenal hyperplasia (17-OH progesterone >2.5 ng/ml), diabetes or treatment with hormones within 3 months prior to the recruitment into the study and/or ovulation induction. Ethical Committees from the Faculty of Medicine and Clinical Hospital from the University of Chile approved this research and all subjects signed an informed consent.

### Tissue preparation

Endometrial tissue samples from the 3 studied groups were divided into 3 fragments. One fragment of each sample was fixed in 4% buffered formaldehyde for 24 h, embedded in paraffin and cut in 4 µm thick sections before histological and immunohistochemical studies. The other 2 fragments of each sample were frozen in liquid nitrogen and stored at -80 °C to measure protein content and gene expression. For protein content, one fragment of frozen samples was lysed in RIPA buffer (50 mM Tris-Base, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X100, and 0.1% sodium dodecyl sulfate), and 1X protease, and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rochester, NY, USA), was added. The samples were centrifuged at 10 000 g for 20 min at 4 °C. The resulting lysate was used to determine protein concentration with the BCA Protein Assay kit (Thermo Fisher Scientific, Rochester, NY, USA).

### Cell culture and treatments

Telomerase-immortalized human endometrial stromal cell lines T-HESC (ATCC, CRL-4003) [25] and St-T1b [26], were cultured in DMEM/Ham F12 medium (Sigma Aldrich Co. Saint Louis, MO, USA) with 10% Fetal Bovine Serum treated with dextran carbon (Hyclone™ Thermo Scientific, NY, USA), and 1X of antimycotic/antibiotic, at 37 °C in a 5% CO<sub>2</sub> atmosphere until 80% confluence. Then, cells were cultured directly on slides (Thermo Scientific™ Nunc™ Lab-Tek™ II Chamber Slide™ System), 50 000 cells/slide in growth media for 24 h at 37 °C in 5% CO<sub>2</sub> atmosphere. The cells were washed twice with sterile Dulbecco's PBS (DPBS, GIBCO® Invitrogen Corporation, Camarillo, CA, USA). The cultures were further subjected for 24 h in serum-free medium to addition of testosterone, insulin, or testosterone plus insulin, 100 nM each. Testosterone and insulin concentrations were determined in previous studies from our laboratory [10,27]. The cultures with no hormonal stimulation were used as basal in each experiment.

### Real-time PCR assay

Frozen endometrial samples were homogenized to obtain total RNA using Trizol reagent according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically (A260:A280), while RNA integrity was determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. RNA was visualized by adding ethidium bromide to the samples. 2 µg of total RNA were digested with DNase I and transcribed into complementary DNA (cDNA) by reverse transcription with M-MLV Reverse Transcriptase by

# Endometria from Obese PCOS Women with Hyperinsulinemia Exhibit Altered Adiponectin Signaling

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## Key words

- adipokine
- adiponectin receptors
- APPL1
- hyperandrogenemia

## Abstract

Hyperandrogenemia, hyperinsulinemia, and obesity affect 60–70% of patients with Polycystic Ovarian Syndrome (PCOS), who exhibit an altered endometrial insulin signaling. The aim of the study was to evaluate whether hyperandrogenism, hyperinsulinism, and obesity present in PCOS patients impair the endometrial adiponectin signaling pathway. The *ex vivo* study was conducted on 27 samples from lean ( $n=9$ ), obese ( $n=9$ ), and obese-PCOS ( $n=9$ ) patients. The *in vitro* assays were performed in immortalized human endometrial stromal cells stimulated with testosterone, insulin, or testosterone plus insulin. Serum steroid-hormones, adiponectin, glucose, and insulin; body mass index, free androgen index, ISI-Composite, and HOMA were evaluated in the 3 groups. *Ex vivo* and *in vitro* gene expression and protein content of adiponectin, AdipoR1, AdipoR2, and APPL1 were

determined. Adiponectin serum levels were decreased in obese-PCOS patients compared to lean (78%) and obese (54%) controls ( $p<0.05$ ). AdipoR1 protein and gene expression were increased in obese group vs. obese-PCOS and lean groups (2-fold,  $p<0.05$ ). In turn, AdipoR2 protein and mRNA content was similar between the 3 groups. APPL1 protein levels were reduced in endometria from both obese groups, compared to lean group (6-fold,  $p<0.05$ ). Testosterone plus insulin stimulation of T-HESC and St-T1b leads to a reduction of adiponectin, AdipoR1, AdipoR2, and APPL1 protein content in both endometrial cell lines ( $p<0.05$ ), whereas, in the presence of testosterone or insulin alone, protein levels were similar to basal. Therefore, endometrial adiponectin-signaling pathway is impaired in hyperandrogenemic and hyperinsulinemic obese-PCOS patients, corroborated in the *in vitro* model, which could affect endometrial function and potentially the implantation process.

received 21.10.2014

accepted 11.06.2015

## Bibliography

DOI <http://dx.doi.org/10.1055/s-0035-1555806>  
 Published online: 2015  
 Horm Metab Res  
 © Georg Thieme Verlag KG  
 Stuttgart · New York  
 ISSN 0018-5043

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## Introduction

Polycystic Ovarian Syndrome (PCOS) is one of the most frequent pathologies related to infertility and recurrent miscarriage. This endocrine and metabolic disease is highly prevalent in women of reproductive age, affecting between 5 and 10% of them [1]. PCOS is characterized by functional hyperandrogenism, as well as ovarian dysfunction represented by a high number of ovarian cysts and/or oligo-anovulation. Also, hyperinsulinemia associated to insulin resistance is present in 50–70% of cases, according to the Rotterdam Consensus [2] and to the Androgen Excess Society criteria [3]. PCOS constitutes a major health problem to women. As a consequence of PCOS, many tissues, besides the ovary, present abnormal functions. These include the skeletal muscle, adipose tissue and, most importantly, the endometrium in its

reproductive function [4]. In fact, several reports have shown that the endometria from PCOS women behave differently from the endometria of normal women: the implantation [5] and cell proliferation/apoptosis processes [6] are disturbed and, also, as it has been recently addressed, the energetic homeostasis, important for metabolic and reproductive endometrial functions, is also abnormal [7]. Thus, impairment of glucose transporter-4 (GLUT4) levels and of proteins related to GLUT4 translocation to the cell surface (WAVE and SNARE protein families) is detected in PCOS derived endometria [8–10]. Meanwhile, disruption of PPAR $\gamma$ /FOXO1 levels and diminished activity of adenosine monophosphate-activated protein kinase (AMPK) is also observed in the pathological endometria [11,12]. Taken together, these data suggest that the correct insulin signaling in the endometrium is important for normal tissue function and that it is disrupted in

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