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Enlarged adipocytes in subcutaneous adipose tissue associated to hyperandrogenism and visceral adipose tissue volume in women with polycystic ovary syndrome

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ABSTRACT

Context: Polycystic ovary syndrome (PCOS) is an androgen excess disorder associated with obesity and adipose tissue disturbances. Our aim was to evaluate gene expression of adipocytokines and adipocyte characteristics in abdominal subcutaneous adipose tissue (SAT) of PCOS women.

Design: Twelve PCOS (PCOSw) and 12 control (Cw) premenopausal women (BMI 20–35 kg/m^2) were included, with measurements of whole-body composition assessed by dual-energy X-ray absorptiometry, and abdominal subcutaneous and visceral adipose tissue (VAT) volume, by magnetic resonance imaging. An oral glucose tolerance test was performed with measurements of glucose and insulin, and sex steroids, lipid profile and serum adipocytokines were determined in the fasting sample. Adipocytokine gene expression, mean adipocyte area and macrophage infiltration were evaluated in SAT biopsies.

Results: Both groups were comparable in age and BMI. Trunk fat mass amount ($p = .043$), serum and SAT leptin/adiponectin ratio ($p = .034$ and $p = .028$, respectively) and adipocyte area ($p = .015$) were higher in PCOSw compared to Cw. Interestingly, trunk fat mass was positively correlated with adipocyte area in PCOSw ($r = 0.821$, $p = .023$), while the inverse correlation was found in Cw ($r = -0.786$, $p = .021$). Only in PCOSw, adipocyte area was positively correlated with serum testosterone ($r = 0.857$, $p = .014$) and visceral adipose tissue volume ($r = 0.857$, $p = .014$).

Conclusions: Our results indicate that PCOS women present adipose tissue dysfunction in the subcutaneous compartment, characterized by an alteration in adipocyte size and leptin/adiponectin expression and secretion, probably associated with higher androgen concentrations.

1. Introduction

Polycystic ovary syndrome (PCOS) is an androgen excess disorder highly prevalent in women of reproductive age, closely associated with metabolic derangements such as insulin resistance and hyperinsulinemia [1]. Women with PCOS are often obese; moreover, around 28% of overweight and obese women have PCOS. Accordingly, obesity plays a preponderant role in the development and severity of PCOS [2]. On the other hand, the hyperandrogenism present in PCOS could also influence the pathophysiology of obesity, favoring the accumulation of adipose tissue and regulating many aspects of its function [2].

The type of adipose tissue depot can vary in its characteristics and function [3]. Although for a long time it was postulated that visceral adipose tissue (VAT) was more relevant than subcutaneous adipose tissue (SAT) in terms of metabolic derangements, currently it is well known that SAT is also linked with metabolic alterations [3,4]. Interestingly, the adipose tissue of PCOS women exhibits enlarged adipocytes, associated with decreased adiponectin production and insulin resistance [5], indicating that alterations in the function and morphology of adipose tissue could be associated with the pathogenesis of PCOS [6].

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The role that androgens could have in adipose tissue has raised special interest, since it has been proposed that in PCOS women many aspects of adipose tissue distribution and function are abnormal, even in the absence of obesity [2]. In this regard, adipose tissue expresses the androgen receptor (AR) in both SAT and VAT depots [7]. Androgens stimulate the growth of adipocytes, disrupt the differentiation of pre-adipocytes to adipocytes, and decrease lipolysis in SAT [8,9]. Although it is not clear, indirect evidence suggests that androgens may be involved in adipose tissue inflammation [8]. Accordingly, we recently demonstrated that exposure of VAT from obese women to testosterone increases monocyte chemoattractant protein-1 production and may, thus, initiate a local inflammatory response [10]. In turn, low adiponectin serum levels and elevated concentrations of inflammatory adipocytokines in women with PCOS suggest the presence of a low-grade chronic inflammation state, however, these findings are still controversial [11,12]. Particularly, adiponectin and leptin have been proposed as markers of adipose tissue function because they are secreted by adipocytes, unlike other cytokines that are also produced by macrophages and stromal cells [13].

Therefore, since body weight and hyperandrogenism are both apparently involved in adipose tissue dysfunction in PCOS, the aim of the present study was to assess the impact of androgens on adipocytokine gene expression and adipocyte size in SAT of PCOS women, compared to non hyperandrogenic women of similar body mass index (BMI) and age.

2. Methods

2.1. Subjects

Twelve premenopausal PCOS women (PCOSw) and 12 non-hyperandrogenic women (control group) between 30 and 52 years of age with a BMI between 20 and 35 kg/m^2, were included in the study. All PCOSw were diagnosed in our Research Unit according to the NIH definition during their early reproductive age. Those women over 40 years who entered this study had been previously diagnosed as PCOS when they participated in other studies of our group. Control women (Cw) were selected from women attending a preventive medical examination at the Department of Obstetrics and Gynecology in our hospital, as previously described [14]. All Cw had a history of regular 28- to 32-day menstrual cycles, absence of hirsutism and other manifestations of hyperandrogenism, and no history of infertility.

None of the PCOS or control women were under hormonal contraceptive therapy or other medication for ≥ 3 months before starting the study. Women with known diabetes, current pregnancy or breast-feeding were excluded.

The protocol was approved by the Institutional Review Boards of the San Juan de Dios Hospital and the University of Chile. All women signed informed consents before entering the study.

2.2. Study protocol

Control and PCOS subjects were studied 3–7 days after menstrual bleeding or whenever feasible in those PCOS women without regular menses. We performed a complete physical examination with anthropometric measurements including: weight, height, waist circumference, hip circumference and waist to hip ratio (WHR), BMI, Ferriman-Gallwey score (scale to assess hirsutism), skinfold thickness and ovarian volume by ultrasound. In all participants, an oral glucose tolerance test (75 g glucose) was performed after a 12-h overnight fast with measurements of glucose and insulin. Insulin resistance was estimated by the homeostasis model assessment for insulin resistance (HOMA-IR) and by the insulin sensitivity index (ISI) composite, and total glucose and insulin secretions were determined as the area under the curve (AUC) calculated by the trapezoidal method using 0-, 30-, 60-, 90- and 120-min time points. Fasting serum concentrations of sex steroids and sex hormone binding globulin (SHBG) were determined, and free androgen index (FAI) was calculated. Additionally, cytokines and lipid profile were assessed.

In the fasting state and under local anesthesia, an abdominal subcutaneous fat biopsy sample from the lower abdomen region was obtained for immunohistochemistry analysis, according to the technique described by Bergström [15]. Additionally, another section of tissue was collected by liposuction aspiration for RNA extraction, and was immediately stored at −80 °C.

Dual X-ray absorptiometry (DXA) was used to measure whole and regional body composition, using a Lunar DPX-L densitometer (Lunar, Madison, WI, software version 1.30). The volumes of SAT and VAT were determined by magnetic resonance imaging (MRI) at the L4–L5 intervertebral disk level with a 1.5 Tesla scanner (Intera, Philips Healthcare).

2.3. Laboratory analyses

Serum testosterone, androstenedione and 17-OHP were assayed by RIA (DIAsource Immunoassay SA, Louvain-la-Neuve, Belgium) and SHBG was determined by IRMA (Institute of Isotops Co. Ltd. (Izotop), Budapest). Sensitivities for testosterone, androstenedione, 17-OHP and SHBG assays were 0.1 ng/ml, 0.1 ng/ml, 0.03 ng/ml and 0.22 nmol/l, respectively. Intra- assay coefficients of variation were 3.5%, 3.8%, 4.6% and 4.9%, and inter-assay coefficients of variation were 5.5%, 7.5%, 7.7% and 3.8%, respectively. Estradiol and estrone were determined by RIA (Pantex, Santa Monica, CA, USA and DIAsource Immunoassay SA, Louvain-la-Neuve, Belgium, respectively). Sensitivities were 10.0 pg/ml and 5.0 pg/ml, respectively, and intra- and inter-assay coefficients of variation were 4.7 and 5.4% for estradiol and 6.4 and 7.1% for estrone.

Glucose, insulin and lipid profile were analyzed as previously described [16]. Serum interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), adiponectin and leptin concentrations were determined by Luminex (Luminex 200, Austin, TX). Intra- and inter-assay variation coefficients were not greater than 2.9% and 5.6%, respectively, for the different metabolites. The detection limit was 0.1 pg/ml for TNF-α, 0.4 pg/ml for IL6, 1.1 pg/ml for MCP-1, 6.0 pg/ml for adiponectin and 4.7 pg/ml for leptin.

2.4. RNA isolation and quantitative real-time RT-PCR

Approximately 100 mg of frozen adipose tissue obtained by liposuction were employed for total RNA extraction using RNeasy Lipid Tissue Mini kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). RNA concentration and purity were evaluated with a Nanodrop ND-1000 spectrophotometer (Nano-Drop Technologies). First-strand cDNA was synthesized from 250 ng of total RNA with Superscript III according to the manufacturer's protocol (SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR, Invitrogen).

Quantitative real-time PCR was performed using a commercial master mix (Taqman Universal PCR Master Mix II, Applied Biosystems) and Taqman probes (Applied Biosystems, Foster City, CA, USA) specific for gene expression of: IL-6 (*IL6*: Hs00985639_m1), TNF-α (*TNFA*: Hs01113624_g1), monocyte chemoattractant protein-1 (*CCL2*: Hs00234140_m1), adiponectin (*ADIPOQ*: Hs00605917_m1), leptin (*LEP*: Hs00174877_m1) and androgen receptor (*AR*: Hs00171172_m1), according to the amplification conditions indicated by the

manufacturer. Samples were run in duplicate in a Stratagene MX3000P thermal cycler (Agilent, USA). Gene expression values were calculated with the ΔΔCq method (RQ = $2^{-\Delta\Delta Cq}$) using *GAPDH* as a normalizer.

2.5. Adipocyte size

Mean adipocyte area was determined in 5 μm sections stained with hematoxylin-eosin. Briefly, images from 3 random fields of each sample were obtained by computerized image analysis with ImageJ software (NIH, USA), with an optical microscope (Leica, LCM2000) attached to a digital camera (Leica, DFC 295) at 40X magnification. One hundred adipocytes were measured and adipocyte area was calculated with the largest diameter in those adipocytes with spherical shape, according to Anand et al. [17]. Mean adipocyte area was calculated with the Ferret formula in ImageJ (http://rsbweb.nih.gov/ij/index.html) and expressed in μm^2.

Table 1
Clinical and biochemical characteristics in control women (Cw) and women with polycystic ovary syndrome (PCOSw).

	Cw (n = 12)	PCOSw (n = 12)	p
Anthropometric and clinical measurements			
Age (years)	42.5 (38.7–43.7)	37.5 (31.0–42.0)	.104
Weight (kg)	64.1 (55.5–70.8)	76.0 (64.2–82.5)	.100
Height (m)	1.55 (1.51–1.62)	1.60 (1.56–1.67)	.099
BMI (kg/m^2)	26.3 (23.7–29.1)	28.5 (26.1–32.6)	.149
WC (cm)	78.0 (71.5–88.2)	87.5 (84.5–95.0)	.049
HC (cm)	98.0 (88.0–107.0)	104.5 (93.5–109.5)	.247
WHR	0.81 (0.78–0.87)	0.87 (0.83–0.93)	.094
Ferriman-Gallwey score	0 (0–2)	14 (10–18)	< .001
Ovarian volume (cc)	4.3 (2.9–5.6)	8.0 (5.4–9.9)	.037
Metabolic measurements			
Fasting			
Glucose (mg/dl)	76.0 (72.2–85.7)	82 (72.2–95.7)	.563
Insulin (μ UI/ml)	10.5 (7.1–12.6)	10.3 (8.9–14.1)	.488
HOMA-IR	1.9 (1.3–2.3)	2.1 (1.8–2.5)	.386
Triglycerides (mg/dl)	162.5 (105.7–187.5)	151.5 (140.2–190.7)	.644
Cholesterol (mg/dl)	203.0(166.7–219.7)	173.0 (159.2–184.0)	.013
HDL-cholesterol (mg/dl)	46.4 (35.6–51.0)	41.2 (33.7–47.0)	.644
LDL-cholesterol (mg/dl)	176.4(156.8–210.3)	165.2 (143.6–174.8)	.033
Post-load			
2 h glucose (mg/dl)	90.0(86.2–118.0)	118.5 (105.7–136.2)	.028
2 h insulin (μUI/ml)	49.5 (35.6–95.1)	98.5 (57.9–159.4)	.057
AUC glucose (mg/dl)	12,660(11,257–15,836)	15,360 (14,047–16,924)	.057
AUC insulin (μUI/ml)	5617(4253–9082)	10,165 (9343–17,890)	.007
ISIcomposite	5.7 (3.8–6.7)	3.5 (2.7–4.1)	.006
Serum sex steroids			
Testosterone (ng/ml)	0.47 (0.34–0.57)	0.56 (0.35–0.77)	.272
Androstenedione (ng/ml)	1.69 (1.14–2.28)	2.77 (1.70–3.51)	.028
Estradiol (pg/ml)	32.62(14.18–59.12)	27.01 (23.76–36.09)	.729
Estrona (pg/ml)	36.60(31.80–50.20)	41.60 (36.00–61.70)	.309
17-OHP (ng/dl)	0.74 (0.49–1.13)	1.31(0.84–1.76)	.149
SHBG (nmol/l)	88.53(50.98–110.57)	49.67 (27.43–89.17)	.057
FAI	2.00 (1.15–3.10)	3.73 (1.25–9.39)	.119
Body composition			
DXA			
Arms tissue fat (%)	35.7 (23.1–42.2)	40.3 (29.8–45.5)	.204
Legs tissue fat (%)	38.6 (30.3–45.6)	35.3 (32.8–38.9)	.583
Trunk tissue fat (%)	41.2 (31.8–44.6)	45.8 (35.2–51.0)	.088
Total tissue fat (%)	40.0 (30.3–41.9)	40.4 (33.4–46.2)	.488
Arms fat (kg)	2.2 (1.2–2.6)	3.1 (2.4–4.0)	.021
Legs fat (kg)	7.9 (5.7–10.5)	8.5 (7.6–9.5)	.488
Trunk fat (kg)	11.7 (8.6–14.1)	15.9 (13.1–19.7)	.043
Total fat (kg)	22.9 (17.2–27.4)	28.2 (24.4–33.3)	.106
Arms lean (kg)	4.0 (3.7–4.5)	4.4 (3.7–5.0)	.386
Legs lean (kg)	12.9 (11.3–14.7)	14.4 (12.1–15.6)	.273
Trunk lean (kg)	19.0 (16.7–20.3)	20.7 (18.2–22.8)	.149
Total lean (kg)	38.8 (35.3–43.0)	44.0 (37.4–45.5)	.149
MRI			
Subcutaneous volume (l)	3.1 (2.7–4.2)	3.6 (2.4–4.9)	.553
Visceral volume (l)	1.2 (0.7–2.0)	1.7 (1.1–2.5)	.166
Visceral/Subcutaneous ratio	0.3 (0.2–0.4)	0.4 (0.3–0.5)	.149

BMI: body mass index, WC: waist circumference, HC: hip circumference, WHR: waist to hip ratio, AUC: area under the curve, SHBG: sex hormone binding globulin, FAI: free androgen index (FAI = [T × 3.467] × 100/SHBG). DXA (dual X-ray absorptiometry), MRI (magnetic resonance imagimg). Data are expressed as median and interquartile range. $p < .05$ was considered significant.

2.6. Immunohistochemistry

Immunohistochemistry was performed on dewaxed 5 µm serial sections. To detect total resident macrophages, pro-inflammatory and anti-inflammatory macrophages, sections were immunostained with specific antibodies: mouse anti human CD68 monoclonal antibody (1:800. Dako. M0814) to detect total macrophages, rabbit anti mouse iNOS polyclonal antibody (1:100. Thermo Fisher. PA1-21,054) and rabbit anti human arginase I polyclonal antibody (1:100. Santa Cruz. H-52) to detect pro-inflammatory and anti-inflammatory macrophages, respectively. Briefly, sections were treated with citrate buffer (10 mM, pH 6.0) for 20 min in a steamer and left to cool at room temperature. Then, they were incubated for 30 min with H_2O_2 and incubated overnight at 4 °C with primary antibody. Corresponding horseradish peroxidase (HRP) enzyme-conjugated anti mouse or rabbit polyclonal antibodies (1:500. Jackson Immuno Research) were incubated for 2 h at room temperature. Finally, sections were counterstained with hematoxylin and mounted with Entellan (Merck). Control experiments were performed in the absence of primary antibodies. Macrophages were counted by double blind procedure using ImageJ software.

2.7. Statistical evaluation

Data are expressed as median and interquartile range. Normality was assessed by the Kolmogorov-Smirnov test, showing a non-normal distribution of the data. Differences between groups were evaluated using the Mann-Whitney test. Spearman correlation analysis was used to evaluate the relationships among variables of interest. The effect of BMI on continuous variables was evaluated using multivariate analysis. Spearman correlation was also adjusted by BMI. The analyses were performed with IBM SPSS 23.0 program. A p-value of $< .05$ was considered statistically significant.

3. Results

3.1. Clinical and biochemical parameters

Clinical and biochemical results are shown in Table 1. Both groups of women were comparable in age, weight, BMI and WHR. Ferriman-Gallwey score, waist circumference and ovarian volume were higher in PCOSw compared to Cw. Regarding metabolic parameters, Cw exhibited higher total and LDL cholesterol compared to PCOSw. PCOSw showed higher post-load glucose concentrations, higher AUC of insulin and lower ISI composite, reflecting higher insulin secretion and lower insulin sensitivity. After adjusting for BMI, only ISI composite remained significantly different (p = .012). Serum androstenedione level was higher in PCOSw compared to Cw. No differences were observed in 17-OHP, testosterone, estrone and estradiol between groups.

3.2. Body composition

Regarding skinfold thickness evaluated by a body caliper, PCOSw exhibited higher suprailiac skinfold compared to Cw (30 [27–36] vs 20 [12–27] mm, p = .033), whereas the abdominal, bicipital, tricipital and subescapular skinfolds were similar between groups. After adjusting for BMI, the suprailiac skinfold remained different between groups (p = .031).

DXA and MRI analyses are shown in Table 1. PCOSw exhibited higher arms and trunk fat mass amount. After adjusting for BMI, these parameters were comparable between groups. Total and regional lean masses were similar between Cw and PCOSw. Finally, MRI showed no differences in VAT and SAT volumes, nor in its ratio in PCOSw compared to Cw.

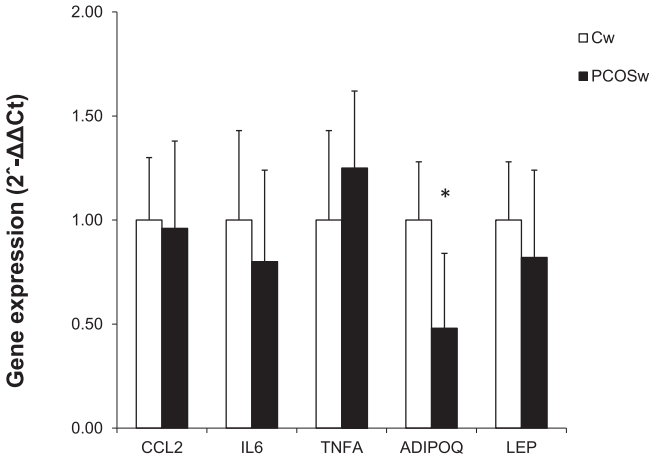

Fig. 1. Adipocytokine messenger RNA expression of subcutaneous adipose tissue in control women (Cw) and women with polycystic ovary syndrome (PCOSw). p < .05 was considered significant.

3.3. Serum adipocytokine levels

There were no differences between Cw and PCOSw in circulating levels of adipocytokines (Table 2). However, the leptin/adiponectin ratio was higher in PCOSw than in Cw (p = .034). After adjusting for BMI, this difference was not significant.

3.4. Gene expression

The relative amount of *ADIPOQ* (p = .033) was lower and the *LEP/ADIPOQ* mRNA ratio (p = .028) was higher in PCOSw compared to Cw. After adjusting for BMI, only this last parameter remained different between groups (p = .027). No differences were observed in the mRNA expression of *IL6*, *LEP*, *CCL2*, *TNFA* or *AR* (Fig. 1).

3.5. Adipose tissue characteristics

Adipose tissue characteristics are shown in Table 2. PCOSw showed a higher mean adipocyte area (p = .015), with adipocytes 49% larger

Table 2
Serum concentration of cytokines and subcutaneous adipose tissue parameters in control women (Cw) and women with polycystic ovary syndrome (PCOSw).

	Cw (n = 12)	PCOSw (n = 12)	p
Serum cytokines			
IL-6 (pg/ml)	3.8 (1.6–4.7)	2.7 (1.9–6.2)	.916
MCP-1 (pg/ml)	308(183–348)	291 (178–368)	.622
TNF (pg/ml)	3.2 (1.5–7.5)	3.9 (2.1–4.3)	.743
Adiponectin (µg/ml)	18.2(9.8–31.4)	12.0 (8.2–17.7)	.123
Leptin (ng/ml)	9.7(6.1–29.6)	18.1 (12.4–22.5)	.250
Leptin/Adiponectin ratio	0.4 (0.3–1.2)	1.1 (1.0–2.1)	.034
Adipocyte parameters			
Adipocyte area (μm^2)	5543(5328–6309)	8235 (6901–8625)	.015
Total macrophages (mm^2)	17 (7–22)	29 (20–38)	.051
Pro-inflammatory macrophages (mm^2)	26 (13–35)	34 (28–43)	.143
Anti-inflammatory macrophages (mm^2)	8 (0–13)	0 (0–19)	.353
LEP/ADIPOQ gene expression ratio	1.07(0.64–1.42)	1.84 (1.08–2.54)	.028
Androgen receptor gene expression (2^{-DDCT})	1.0 (0.8–1.3)	0.7 (0.5–0.9)	.214

Data are expressed as median and interquartile range. p < .05 was considered significant.

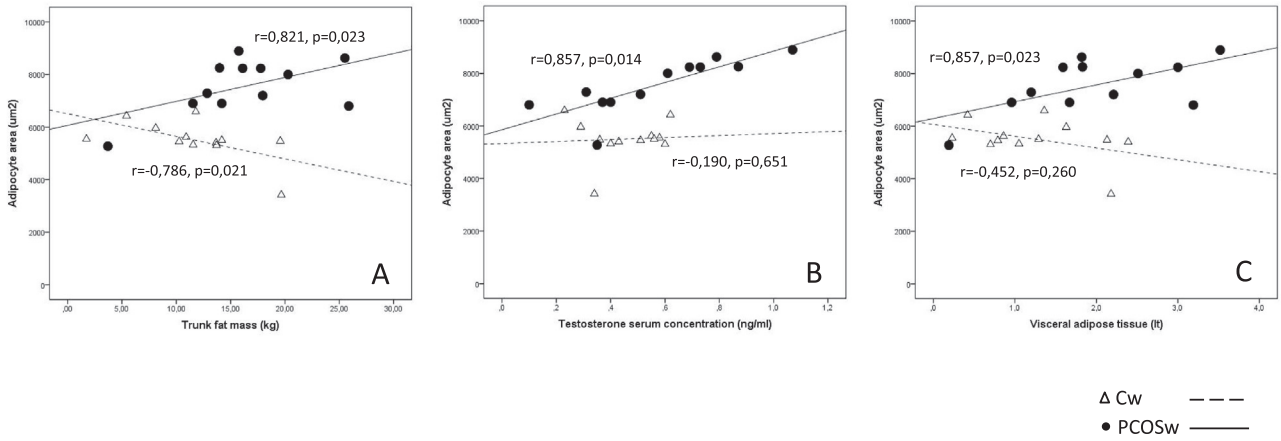

Fig. 2. Bivariate correlations between adipocyte area of subcutaneous adipose tissue and trunk fat mass (A), testosterone serum concentration (B) and visceral adipose tissue (C) in control women (Cw) and women with polycystic ovary syndrome (PCOSw). r: Spearman's correlation coefficient. $p < .05$ was considered significant.

than Cw. Moreover, there was a trend to higher density of total macrophages (CD68 positive) in the PCOS group ($p = .051$), without differences in proinflammatory or anti-inflammatory macrophages. After adjusting for BMI, adipocyte area and density of total macrophages were significantly higher in PCOSw ($p = .004$ and $p = .015$, respectively).

3.6. Correlation analysis

In PCOSw, adiponectin serum concentration was positively correlated with adiponectin gene expression in SAT ($r = 0.609$, $p = .047$), and negatively correlated with AUC of insulin (-0.791, $p = .004$); these correlations were not found in Cw. No correlation between serum concentration and gene expression was observed in the other adipocytokines.

In both groups of women, BMI was positively associated with trunk fat mass amount evaluated by DXA ($r = 0.860$, $p < .001$ in Cw and $r = 0.769$, $p = .003$ in PCOSw). In this regard, only in Cw, BMI and trunk fat mass were positively correlated with both SAT and VAT volumes. We found no correlation between leptin or adiponectin serum levels and BMI or parameters of body adiposity in Cw or PCOSw. Nevertheless, in PCOSw trunk fat mass was negatively correlated with adiponectin gene expression, which was not maintained after correction by BMI.

Interestingly, SAT mean adipocyte area was positively correlated with trunk fat mass in PCOSw and negatively in Cw (Fig. 2). Both correlations were lost after correction by BMI. Moreover, mean adipocyte area was positively correlated with testosterone serum concentration and with VAT volume evaluated by MRI only in PCOSw (Fig. 2). These correlations were maintained after correction by BMI ($p = .042$ and $p = .039$, respectively).

4. Discussion

In the present study, we analyzed the morphology and physiology of SAT in women with PCOS, finding an increased leptin to adiponectin ratio in serum and in tissue, more infiltration of macrophages and enlarged adipocytes. The latter correlated with the degree of hyperandrogenism and with the amount of VAT.

In general, it has been observed that SAT is more important than VAT in adipocytokine production [18]. Therefore, it is likely that SAT could be the main responsible for levels of circulating adipokines, especially adiponectin. Similar to that found by Lecke et al. [19], we observed a positive correlation between serum concentrations and mRNA levels of adiponectin in tissue. Therefore, we confirmed that SAT production of adiponectin seems to be the main determinant of serum adiponectin levels, as previously described [20].

Circulating leptin to adiponectin ratio has been described as a good marker of the inflammatory state in PCOS and a better reflection of the adipocyte secretory function than isolated adipokine levels [21]. We observed that this parameter was increased in both SAT and serum from PCOSw. Interestingly, in tissue this association was independent of BMI, while in circulation it was influenced by BMI, indicating that circulating concentrations of adiponectin and leptin can also be modulated by post-transcriptional mechanisms and by local environment [4]. In turn, the lower mRNA expression of *ADIPOQ* without differences in the gene expression of *LEP*, *IL6*, *CCL2* or *TNFA* in SAT of women with PCOS, observed in the present study and in previous ones [12], indicates that gene expression of adiponectin rather than other adipocytokines is influenced by the PCOS condition.

It has been reported that variations in fat cell size in obesity may have an important impact on adipose tissue function [22]. Our study shows that SAT of women with PCOS has enlarged adipocytes, with a mean area 49% greater than controls. In this regard, it has been proposed that under metabolic stressors, such as obesity and androgen excess, the process of adipose tissue expansion is altered causing adipocyte hypertrophy [23]. According to this observation, we found that the adipocyte area was positively correlated with testosterone serum concentrations but not with body weight in PCOS women. Androgens do not modify preadipocyte proliferation but they do inhibit their differentiation [24]. In this regard, it has been observed that androgens inhibit adipocyte differentiation [24] but promote their growth [3], contributing to the process of adipose tissue expansion [25]. This observation is probably valid within certain conditions, because androgens have a pleiotropic effect depending on androgen levels and body weight [26].

In addition to the effect of androgens on adipose tissue, estrogens also influence the distribution and function of adipose tissue through binding to their specific receptors [27,28]. Adipose tissue is considered to be the major source of circulating estrogens after the gonads, and the contribution made by adipose tissues to total circulating estrogens increases with advancing age and weight [29,30]. The conversion of androgens to estrogen is mediated by aromatase, a cytochrome P450 enzyme; this process occurs in extragonadal tissues [31]. While in the reproductive period estradiol is the predominant estrogen, during the perimenopause estrone is the main estrogen [32]. Wang et al. inferred that an imbalance between androgens and estrogens observed in the PCOS condition could disturb the normal function and enzyme gene expression of adipose tissue [33], reaching mildly masculinized characteristics [34,35]. In the present study, estradiol and estrone serum concentrations were similar in women with and without PCOS. However, it cannot be ruled out that in the adipose tissue, some difference could be observed. In fact, it has been observed that estrone concentration in adipose tissue is several times higher than its circulating levels [36–38].

SAT has a limited capacity to increase its mass safely. When it reaches its maximum capacity, there is an imbalance with accumulation in non-SAT and other organs and tissues; among them, VAT could be affected. This is known as the hypothesis of adipose tissue expandability. The concept implies that there is a "metabolic setpoint", which could be affected by high concentrations of insulin and androgens [39]. Then, individuals with larger adipocytes in SAT have a lower capacity for further lipid storage, so the subsequent excess of fat may be stored in VAT, liver and skeletal muscle [40] with the consequent development of metabolic disorders [41]. In this sense, in our study we observed that only in the PCOS there was a positive correlation between size of subcutaneous adipocytes and visceral fat content, which supports the aforementioned phenomenon.

It is interesting to mention that hypertrophic adipocytes are more susceptible to inflammation, apoptosis, fibrosis and release of free fatty acids, and are associated with higher macrophage infiltration [11,42]. In this context, we found that the total macrophage density in SAT was higher in women with PCOS independent of BMI. Although there is still no consensus on this issue, a recent study demonstrated that PCOS is associated with an increase in the density of macrophages, especially of a proinflammatory phenotype. We did not observe significant differences in pro- or anti-inflammatory macrophage density, probably due to the sample size.

Regarding adipocyte function, it has been suggested that large adipocytes are characterized by reduced production and secretion of adiponectin compared with small newly differentiated adipocytes [43]. Manneras-Holm et al. found enlarged adipocytes in Swedish PCOSw associated with reduced adiponectin secretion and lower insulin sensitivity [5]. In our study, mean adipocyte size was not correlated with serum adiponectin concentrations. However, both increased mean adipocyte size and low adiponectin expression were found in PCOS women.

Apparently, body fat distribution is even more important than total amount of fat in terms of developing metabolic complications. Regarding fat distribution, we observed higher trunk fat mass accumulation in women with PCOS evaluated by DXA. Although this method is not able to distinguish between SAT and VAT, both contributions are metabolically deleterious [44]. Moreover, we did not find differences in SAT or VAT volume evaluated by MRI between PCOS and control women, similar to that observed by Barber et al. in obese British/Irish premenopausal women with and without PCOS [45]. It has been reported that in women with PCOS, there is an increased accumulation of adipose tissue in abdominal depots [6], predisposing them to the development of metabolic diseases such as IR and diabetes [46] or to a higher risk for metabolic sequelae [44]. Although in both control and PCOS women, BMI was positively associated with trunk fat mass amount, we observed different characteristics of the SAT in each group. In PCOSw, there was a positive correlation between trunk fat mass amount and size of subcutaneous adipocytes; on the contrary in control women, trunk fat mass was negatively associated with adipocyte size. These observations suggest that the mechanism behind obesity in PCOS women is related with hypertrophy of adipose cells rather than with hyperplasia, as might be occurring in control women [5]. In this context, it has been suggested that depot differences in the degree of hypertrophic vs. hyperplastic expansion of adipose tissue in obesity, have an impact on adipose tissue function [47], accounting for a different regulation between women with and without PCOS which could be modulated by a different microenvironment, by changes in the enzymatic pathways and by a genetic and epigenetic background.

Differential expansion and protein expression between SAT and VAT are mutually regulated and probably in normal conditions they remain in balance. As stated before, in women with PCOS we found a positive correlation between the size of subcutaneous adipocytes and both the amount of VAT and the serum testosterone level, which together could be interpreted as follows: testosterone promotes the growth of mature subcutaneous adipocytes to their maximum potential and impairs the differentiation of pre-adipocytes to new mature adipocytes, then VAT may have to expand to take care of the increase in lipid overload not managed by SAT and finally divert triglycerides towards the liver and portal circulation [48]. The higher volume of VAT may be the result of a limited expansion or overload of SAT in PCOS women. Therefore, some of the characteristics of the subcutaneous and visceral adipose tissues in PCOS, as well as the way in which both tissues are interrelated, could be attributed, in part, to sex steroids and obesity.

Hyperinsulinism and dyslipidemia are characteristics of the PCOS condition [49–51]. In the present study, PCOSw showed higher insulin secretion and lower insulin sensitivity; however, Cw exhibited higher total and LDL cholesterol levels compared to PCOSw. It has been demonstrated that the PCOS condition presents an intrinsic insulin resistance which is independent of BMI [52]. However, the lipid profile depends on other factors such as eating habits and physical exercise. In this regard, in the Chilean population obesity is reaching values over 60% in adulthood, mainly due to an increase in poor eating habits coupled with a very sedentary lifestyle [53]. On the other hand, many of the PCOS women are more aware of the disease and its deleterious consequences, so they care more about their nutrition.

A limitation of this study is the reduced number of participants, mainly due to a low rate of adherence to the biopsy procedure. However, within the advantages is that both groups of women have similar age and BMI, were diagnosed in our laboratory with the same criteria in their early reproductive age.

5. Conclusion

Our results show that, SAT from premenopausal PCOS women is dysfunctional, showing an imbalance in expression and secretion between leptin and adiponectin, enlarged adipocytes and increased macrophage infiltration. In a novel way, we found that the increased mean adipocyte size in SAT was associated with higher serum testosterone concentration, VAT volume and trunk fat mass amount, probably reflecting a reduced expandability of SAT caused by androgens and increasing the burden on VAT. These findings suggest that androgens contribute to adipose tissue dysfunction and to metabolic derangements of PCOS.

Authors' contributions

B. E., N.C. and M.M. analyzed the data and wrote the manuscript. J.E.G., D.S., C.S. and N.C. participated in data collection and analysis. B.E. and M.M. performed the statistical analysis. T.S-P. and F. P-B. designed and conducted the study.

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Conflicts of interest

The authors have no conflicts of interest.

References

[1] R.J. Norman, D. Dewailly, R.S. Legro, T.E. Hickey, Polycystic ovary syndrome, Lancet 370 (9588) (2007) 685–697.
[2] T.M. Barber, M.I. McCarthy, J.A. Wass, S. Franks, Obesity and polycystic ovary syndrome, Clin. Endocrinol. 65 (2) (2006) 137–145.
[3] M. Corton, J.I. Botella-Carretero, A. Benguria, G. Villuendas, A. Zaballos, J.L. San Millan, H.F. Escobar-Morreale, B. Peral, Differential gene expression profile in omental adipose tissue in women with polycystic ovary syndrome, J. Clin.

Endocrinol. Metab. 92 (1) (2007) 328–337.
[4] J.N. Fain, A.K. Madan, M.L. Hiler, P. Cheema, S.W. Bahouth, Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans, Endocrinology 145 (5) (2004) 2273–2282.
[5] L. Manneras-Holm, H. Leonhardt, J. Kullberg, E. Jennische, A. Oden, G. Holm, M. Hellstrom, L. Lonn, G. Olivecrona, E. Stener-Victorin, M. Lonn, Adipose tissue has aberrant morphology and function in PCOS: enlarged adipocytes and low serum adiponectin, but not circulating sex steroids, are strongly associated with insulin resistance, J. Clin. Endocrinol. Metab. 96 (2) (2011) E304–E311.
[6] S. Sam, Adiposity and metabolic dysfunction in polycystic ovary syndrome, Hormone Mole. Biol. Clin. Invest. 21 (2) (2015) 107–116.
[7] M.N. Dieudonne, R. Pecquery, A. Boumediene, M.C. Leneveu, Y. Giudicelli, Androgen receptors in human preadipocytes and adipocytes: regional specificities and regulation by sex steroids, Am. J. Physiol. 274 (6 Pt 1) (1998) C1645–C1652.
[8] K. Blouin, A. Veilleux, V. Luu-The, A. Tchernof, Androgen metabolism in adipose tissue: recent advances, Mole. Cell. Endocrinol. 301 (1–2) (2009) 97–103.
[9] G. Chazenbalk, P. Singh, D. Irge, A. Shah, D.H. Abbott, D.A. Dumesic, Androgens inhibit adipogenesis during human adipose stem cell commitment to preadipocyte formation, Steroids 78 (9) (2013) 920–926.
[10] N. Crisosto, C. Flores, M. Maliqueo, B. Echiburu, J. Vasquez, F. Maluenda, T. Sir-Petermann, Testosterone increases CCL-2 expression in visceral adipose tissue from obese women of reproductive age, Mole. Cell. Endocrinol. 444 (2017) 59–66.
[11] H.F. Escobar-Morreale, M. Luque-Ramirez, F. Gonzalez, Circulating inflammatory markers in polycystic ovary syndrome: a systematic review and metaanalysis, Fertil Steril 95 (3) (2011) 1048–1058 e1-2.
[12] A. Lindholm, C. Blomquist, M. Bixo, I. Dahlbom, T. Hansson, I. Sundstrom Poromaa, J. Buren, No difference in markers of adipose tissue inflammation between overweight women with polycystic ovary syndrome and weight-matched controls, Hum. Reprod. 26 (6) (2011) 1478–1485.
[13] J. Vrbikova, K. Dvorakova, M. Hill, J. Vcelak, S. Stanicka, M. Vankova, D. Sramkova, K. Vondra, B. Bendlova, L. Starka, Determinants of circulating adiponectin in women with polycystic ovary syndrome, Gynecol. Obstet. Invest. 60 (3) (2005) 155–161.
[14] A.L. de Guevara, N. Crisosto, B. Echiburu, J. Preisler, N. Vantman, J. Bollmann, F. Perez-Bravo, T. Sir-Petermann, Evaluation of ovarian function in 35–40-year-old women with polycystic ovary syndrome, Eur. J. Obstetr., Gynecol., Reprod. Biol. 170 (1) (2013) 165–170.
[15] J. Bergstrom, Percutaneous needle biopsy of skeletal muscle in physiological and clinical research, Scandinavian J. Clin. Lab. Invest. 35 (7) (1975) 609–616.
[16] B. Echiburu, N. Crisosto, M. Maliqueo, F. Perez-Bravo, A.L. de Guevara, P. Hernandez, G. Cavada, C. Rivas, A. Clavel, T. Sir-Petermann, Metabolic profile in women with polycystic ovary syndrome across adult life, Metabolism, Clin. Exp. 65 (5) (2016) 776–782.
[17] S.S. Anand, M.A. Tarnopolsky, S. Rashid, K.M. Schulze, D. Desai, A. Mente, S. Rao, S. Yusuf, H.C. Gerstein, A.M. Sharma, Adipocyte hypertrophy, fatty liver and metabolic risk factors in South Asians: the Molecular Study of Health and Risk in Ethnic Groups (mol-SHARE), PLoS One 6 (7) (2011) e22112.
[18] P.A. Vardhana, C. Dicken, D.V. Tortoriello, M. Chu, E. Carmina, R.A. Lobo, Increasing adiposity in normal ovulatory women affects adipocytokine expression in subcutaneous and visceral abdominal fat, Int. J. Gynaecol. Obstetr. 104 (2) (2009) 121–124.
[19] S.B. Lecke, D.M. Morsch, P.M. Spritzer, Association between adipose tissue expression and serum levels of leptin and adiponectin in women with polycystic ovary syndrome, Genet. Mol. Res. 12 (4) (2013) 4292–4296.
[20] S.B. Lecke, F. Mattei, D.M. Morsch, P.M. Spritzer, Abdominal subcutaneous fat gene expression and circulating levels of leptin and adiponectin in polycystic ovary syndrome, Fertil Steril 95 (6) (2011) 2044–2049.
[21] N. Xita, I. Papassotiriou, I. Georgiou, M. Vounatsou, A. Margeli, A. Tsatsoulis, The adiponectin-to-leptin ratio in women with polycystic ovary syndrome: relation to insulin resistance and proinflammatory markers, Metabolism, Clin. Exp. 56 (6) (2007) 766–771.
[22] E. Arner, P.O. Westermark, K.L. Spalding, T. Britton, M. Ryden, J. Frisen, S. Bernard, P. Arner, Adipocyte turnover: relevance to human adipose tissue morphology, Diabetes 59 (1) (2010) 105–109.
[23] T.M. Barber, S. Franks, Adipocyte biology in polycystic ovary syndrome, Mole. Cell. Endocrinol. 373 (1–2) (2013) 68–76.
[24] K. Blouin, M. Nadeau, M. Perreault, A. Veilleux, R. Drolet, P. Marceau, J. Mailloux, V. Luu-The, A. Tchernof, Effects of androgens on adipocyte differentiation and adipose tissue explant metabolism in men and women, Clini. Endocrinol. 72 (2) (2010) 176–188.
[25] R. Taylor, R.R. Holman, Normal weight individuals who develop type 2 diabetes: the personal fat threshold, Clin. Sci. 128 (7) (2015) 405–410.
[26] F. Gonzalez, Inflammation in polycystic ovary syndrome: underpinning of insulin resistance and ovarian dysfunction, Steroids 77 (4) (2012) 300–305.
[27] S. Rodriguez-Cuenca, M. Monjo, A.M. Proenza, P. Roca, Depot differences in steroid receptor expression in adipose tissue: possible role of the local steroid milieu, Am. J. physiol. Endocrinol. Metab. 288 (1) (2005) E200–E207.
[28] M. Luque-Ramirez, M.A. Martinez-Garcia, R. Montes-Nieto, E. Fernandez-Duran, M. Insenser, M. Alpanes, H.F. Escobar-Morreale, Sexual dimorphism in adipose tissue function as evidenced by circulating adipokine concentrations in the fasting state and after an oral glucose challenge, Hum. Reprod. 28 (7) (2013) 1908–1918.
[29] P.C. MacDonald, C.D. Edman, D.L. Hemsell, J.C. Porter, P.K. Siiteri, Effect of obesity on conversion of plasma androstenedione to estrone in postmenopausal women with and without endometrial cancer, Am. J. Obstetr. Gynecol. 130 (4) (1978) 448–455.
[30] D.L. Hemsell, J.M. Grodin, P.F. Brenner, P.K. Siiteri, P.C. MacDonald, Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age, J. Clin. Endocrinol. Metab. 38 (3) (1974) 476–479.
[31] E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, M.M. Hinshelwood, S. Graham-Lorence, B. Amarneh, Y. Ito, C.R. Fisher, M.D. Michael, et al., Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, Endocr. Rev. 15 (3) (1994) 342–355.
[32] E.R. Simpson, Sources of estrogen and their importance, J. Steroid Biochem. Mole. Biol. 86 (3–5) (2003) 225–230.
[33] L. Wang, S. Li, A. Zhao, T. Tao, X. Mao, P. Zhang, W. Liu, The expression of sex steroid synthesis and inactivation enzymes in subcutaneous adipose tissue of PCOS patients, J. Steroid Biochem. Mole. Biol. 132 (1–2) (2012) 120–126.
[34] M.A. Martinez-Garcia, R. Montes-Nieto, E. Fernandez-Duran, M. Insenser, M. Luque-Ramirez, H.F. Escobar-Morreale, Evidence for masculinization of adipokine gene expression in visceral and subcutaneous adipose tissue of obese women with polycystic ovary syndrome (PCOS), J. Clin. Endocrinol. Metab. 98 (2) (2013) E388–E396.
[35] S. Borruel, E. Fernandez-Duran, M. Alpanes, D. Marti, F. Alvarez-Blasco, M. Luque-Ramirez, H.F. Escobar-Morreale, Global adiposity and thickness of intraperitoneal and mesenteric adipose tissue depots are increased in women with polycystic ovary syndrome (PCOS), J. Clin. Endocrinol. Metab. 98 (3) (2013) 1254–1263.
[36] J.P. Deslypere, L. Verdonck, A. Vermeulen, Fat tissue: a steroid reservoir and site of steroid metabolism, J. Clin. Endocrinol. Metab. 61 (3) (1985) 564–570.
[37] J. Szymczak, A. Milewicz, J.H. Thijssen, M.A. Blankenstein, J. Daroszewski, Concentration of sex steroids in adipose tissue after menopause, Steroids 63 (5–6) (1998) 319–321.
[38] T. Kinoshita, S. Honma, Y. Shibata, K. Yamashita, Y. Watanabe, H. Maekubo, M. Okuyama, A. Takashima, N. Takeshita, Aninnovative LC-MS/MS-based method for determining CYP 17 and CYP 19 activity in the adipose tissue of pre- and postmenopausal and ovariectomized women using 13C-labeled steroid substrates, J. Clin. Endocrinol. Metab. 99 (4) (2014) 1339–1347.
[39] F. de Zegher, A. Lopez-Bermejo, L. Ibanez, Adipose tissue expandability and the early origins of PCOS, Trends Endocrinol. Metab.: TEM 20 (9) (2009) 418–423.
[40] E. Danforth Jr., Failure of adipocyte differentiation causes type II diabetes mellitus? Nat. Genetics 26 (1) (2000) 13.
[41] B.H. Goodpaster, S. Krishnaswami, T.B. Harris, A. Katsiaras, S.B. Kritchevsky, E.M. Simonsick, M. Nevitt, P. Holvoet, A.B. Newman, Obesity, regional body fat distribution, and the metabolic syndrome in older men and women, Archives Internal Med. 165 (7) (2005) 777–783.
[42] E. Carmina, M.C. Chu, C. Moran, D. Tortoriello, P. Vardhana, G. Tena, R. Preciado, R. Lobo, Subcutaneous and omental fat expression of adiponectin and leptin in women with polycystic ovary syndrome, Fertil Steril 89 (3) (2008) 642–648.
[43] X. Yang, P.A. Jansson, I. Nagaev, M.M. Jack, E. Carvalho, K.S. Sunnerhagen, M.C. Cam, S.W. Cushman, U. Smith, Evidence of impaired adipogenesis in insulin resistance, Biochem. Biophys. Res. Commun. 317 (4) (2004) 1045–1051.
[44] E. Carmina, S. Bucchieri, A. Esposito, A. Del Puente, P. Mansueto, F. Orio, G. Di Fede, G. Rini, Abdominal fat quantity and distribution in women with polycystic ovary syndrome and extent of its relation to insulin resistance, J. Clin. Endocrinol. Metab. 92 (7) (2007) 2500–2505.
[45] T.M. Barber, S.J. Golding, C. Alvey, J.A. Wass, F. Karpe, S. Franks, M.I. McCarthy, Global adiposity rather than abnormal regional fat distribution characterizes women with polycystic ovary syndrome, J. Clin. Endocrinol. Metab. 93 (3) (2008) 999–1004.
[46] H.F. Escobar-Morreale, J.L. San Millan, Abdominal adiposity and the polycystic ovary syndrome, Trends Endocr. Metab.: TEM 18 (7) (2007) 266–272.
[47] L.K. Meyer, T.P. Ciaraldi, R.R. Henry, A.C. Wittgrove, S.A. Phillips, Adipose tissue depot and cell size dependency of adiponectin synthesis and secretion in human obesity, Adipocyte 2 (4) (2013) 217–226.
[48] S. Virtue, A. Vidal-Puig, It's not how fat you are, it's what you do with it that counts, PLoS Biol. 6 (9) (2008) e237.
[49] R.S. Legro, A.R. Kunselman, A. Dunaif, Prevalence and predictors of dyslipidemia in women with polycystic ovary syndrome, Am. J. Med. 111 (8) (2001) 607–613.
[50] J.J. Kim, Y.M. Choi, Dyslipidemia in women with polycystic ovary syndrome, Obstetr. Gynecol. Sci. 56 (3) (2013) 137–142.
[51] E. Diamanti-Kandarakis, P.M. Spritzer, T. Sir-Petermann, A.B. Motta, Insulin resistance and polycystic ovary syndrome through life, Curr. Pharmaceutical Des. 18 (34) (2012) 5569–5576.
[52] N.K. Stepto, S. Cassar, A.E. Joham, S.K. Hutchison, C.L. Harrison, R.F. Goldstein, H.J. Teede, Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic-hyperinsulaemic clamp, Hum. Reprod. 28 (3) (2013) 777–784.
[53] Ministerio de Salud del Gobierno de Chile, Encuesta Nacional de Salud ENS 2009-2010, Ministerio de Salud, 2011.