科技部補助專題研究計畫成果報告
期末報告

台灣女性血清中發炎標識、氧化壓標識及環境荷爾蒙標識對月經週期及女生殖荷爾蒙的影響

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 102-2629-B-038-001-
執 行 期 間 ：102 年 08 月 01 日至 103 年 07 月 31 日
執 行 單 位 ：臺北醫學大學婦產科

計 畫 主 持 人：徐明義
共 同 主 持 人：王靜瓊、許淳森、曾啟瑞、黃士懿、陳亦仁
計畫參與人員：碩士級-專任助理人員：徐婉晴
學士級-專任助理人員：陳可倪

處 理 方 式 ：
1.公開資訊：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢
2.「本研究」是否已有嚴重損及公共利益之發現：否
3.「本報告」是否建議提供政府單位施政參考：否

中 華 民 國 103 年 10 月 30 日

中 文 摘 要 ： 研究目的
探討女性的內分泌失調和代謝症候群之間的關聯性並尋求對婦女得妥善醫療照顧

研究方法
本研究為一回溯性研究，以集群分析方式分析。共納入 573 位女性，並分成 384 位低風險和 189 位高風險之心血管疾病。

研究結果
研究發現女性初經來得越早、高濃度的 C 反應蛋白質及肝臟酵素和低濃度的性激素結合蛋白（ sex hormone-binding globulin，SHBG ）都與代謝症候群的危險因子有關。此外，過重或肥胖、寡經或無經症以及高雄性激素症是會提高心血管-代謝疾病的風險因子。不過，高泌乳激素和過早的卵巢功能失調則和心血管-代謝疾病無關。以雄性激素方面而論，血清中的睪丸酮和游離雄性激素指數（ free androgen index ）與心血管-代謝疾病有關。

研究結論
總結所得的結果，雖然多囊性卵巢症候群和代謝疾病有關，但在生育年齡婦女中肥胖是心血管-代謝疾病的主要危險因子。

中文關鍵詞： 心血管風險、代謝症候群、多囊性卵巢症候群、集群分析

英 文 摘 要 ： Objective: To study the association between endocrine disturbances and metabolic complications in women seeking gynecologic care.
Design: Retrospective study, cluster analysis
Setting: Outpatient clinic, University Medical Center
Patients: A total of 573 subjects, including 384 at low risk and 189 at high risk of cardio-metabolic disease, were evaluated.
Interventions: None
Main Outcome Measure(s): Cardiovascular and metabolic parameters and clinical and biochemical characteristics.
Results: Risk factors for metabolic disease are associated with a low age of menarche, high levels of high-sensitivity C-reactive protein and liver

enzymes, and low levels of sex hormone-binding globulin. Overweight/obesity (odds ratio: 11.2； 95% confidence interval: 8.0-15.7), PCOS (1.6； 1.3-1.9), oligo/amenorrhea (1.3, 1.1-1.4), and hyperandrogenism (1.4, 1.2-1.6) were found to increase the risk of cardio-metabolic disease. However, hyperprolactinemia and premature ovarian failure were not associated with the risk of cardio-metabolic disease. In terms of androgens, the serum total testosterone level and free androgen index, but not androstenedione or DHEAS, were associated with cardio-metabolic risk. Conclusions: Although PCOS is associated with metabolic risk, obesity was the major determinant of cardio-metabolic disturbances in reproductive aged women. Hyperprolactinemia and premature ovarian failure were not associated with the risk of cardiovascular and metabolic diseases.

英文關鍵詞： cardiovascular risk, metabolic syndrome, PCOS, cluster analysis

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

台灣女性血清中發炎標識、氧化壓標識及環境荷爾蒙標識對月經週期及女生殖荷爾蒙的影響

計畫類別：■個別型計畫　□整合型計畫
計畫編號：MOST 102-2629-B-038-001
執行期間：2013 年 8 月 1 日至 2014 年 7 月 31 日

執行機構及系所：臺北醫學大學婦產科

計畫主持人：徐明義
共同主持人：王靜瓊、許淳森、曾啟瑞、黃士懿、陳亦仁
計畫參與人員：徐明義、王靜瓊、許淳森、曾啟瑞、黃士懿、陳亦仁

期末報告處理方式：

1. 公開方式：
   □非列管計畫亦不具下列情形，立即公開查詢
   □涉及專利或其他智慧財產權，□一年□二年後可公開查詢
2.「本研究」是否已有嚴重損及公共利益之發現：□否 □是
3.「本報告」是否建議提供政府單位施政參考 □否 □是，____（請列舉提供之單位；本部不經審議，依勾選逕予轉送）

中　華　民　國　103　年 10 月 22 日

目錄

中文摘要

心血管和代謝危險因子在生育年齡婦女之集群分析

曾啟瑞[1]，張源俊[2]，張祐嘉[2]，王家瑋[1]，陳啟煌[1]，徐明義[3]

[1]臺北醫學大學附設醫院婦產科
[2]中央研究院統計科學研究所
[3]臺北市立萬芳醫院-委託財團法人臺北醫學大學辦理婦產科

研究目的
探討女性的內分泌失調和代謝症候群之間的關聯性並尋求對婦女得妥善醫療照顧

研究方法
本研究為一回溯性研究，以集群分析方式分析。共納入 573 位女性，並分成 384 位低風險和 189 位高風險之心血管疾病。

研究結果
研究發現女性初經來得越早、高濃度的 C 反應蛋白質及肝臟酵素和低濃度的性激素結合蛋白（sex hormone-binding globulin，SHBG）都與代謝症候群的危險因子有關。此外，過重或肥胖、寡經或無經症以及高雄性激素症是會提高心血管-代謝疾病的風險因子。不過，高泌乳激素和過早的卵巢功能失調則和心血管-代謝疾病無關。以雄性激素方面而論，血清中的睪丸酮和游離雄性激素指數（free androgen index）與心血管-代謝疾病有關。

研究結論
總結所得的結果，雖然多囊性卵巢症候群和代謝疾病有關，但在生育年齡婦女中肥胖是心血管-代謝疾病的主要危險因子。

關鍵詞：心血管風險、代謝症候群、多囊性卵巢症候群、集群分析

Abstract

Cluster analysis of cardiovascular and metabolic risk factors in women of reproductive age

Chii-Ruey Tzeng, MD[1], Yuan-chin Ivan Chang PhD[2], Yu-chia Chang MSc[2], Chia-Woei Wang, MD[1], Chi-Huang Chen MD, PhD[1], Ming-I Hsu MD[3]

[1]Department of Obstetrics and Gynecology, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan

[2] Institute of Statistical Science, Academia Sinica, Taipei, Taiwan

[3]Department of Obstetrics and Gynecology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan.

Objective: To study the association between endocrine disturbances and metabolic complications in women seeking gynecologic care.

Design: Retrospective study, cluster analysis

Setting: Outpatient clinic, University Medical Center

Patients: A total of 573 subjects, including 384 at low risk and 189 at high risk of cardio-metabolic disease, were evaluated.

Interventions: None

Main Outcome Measure(s): Cardiovascular and metabolic parameters and clinical and biochemical characteristics.

Results: Risk factors for metabolic disease are associated with a low age of menarche, high levels of high-sensitivity C-reactive protein and liver enzymes, and low levels of sex hormone-binding globulin. Overweight/obesity (odds ratio: 11.2; 95% confidence interval: 8.0-15.7), PCOS (1.6; 1.3-1.9), oligo/amenorrhea (1.3, 1.1-1.4), and hyperandrogenism (1.4, 1.2-1.6) were found to increase the risk of cardio-metabolic disease. However, hyperprolactinemia and premature ovarian failure were not associated with the risk of cardio-metabolic disease. In terms of androgens, the serum total testosterone level and free androgen index, but not androstenedione or DHEAS, were associated with cardio-metabolic risk.

Conclusions: Although PCOS is associated with metabolic risk, obesity was the major determinant of cardio-metabolic disturbances in reproductive aged women. Hyperprolactinemia and premature ovarian failure were not associated with the risk of cardiovascular and metabolic diseases.

Keywords: cardiovascular risk, metabolic syndrome, PCOS, cluster analysis

報告內容

Introduction

A cluster of risk factors for cardiovascular disease (CVD) and type 2 diabetes mellitus, which occur together more often than by chance, have become known as metabolic syndrome (MetS) (1). The cardiovascular risk factors that comprise MetS have been recognized as a cluster since the 1920s (2). Although MetS and CVD are major causes of mortality for women of advanced age, the risks of MetS and CVD in reproductive age women are not well understood. The early detection of individuals at high risk for MetS using accurate measures of insulin resistance (IR) could improve the detection and prevention of CVD and diabetes (3). Recent studies suggest that there are some clinically relevant differences between women and men in terms of the prevalence, presentation, management and outcomes of the disease, but little is known about why CVD affects women and men differently (4). Over recent decades, mortality rates in men have steadily declined, while those in women have remained stable. This knowledge gap may explain why cardiovascular health in women is not improving as fast as that of men (4). In particular, the risk of developing MetS and CVD for younger women has not been well studied. Although many aspects of CVD are similar in women and men, there is a growing body of evidence to support sex and gender dimorphisms in the prevalence, presenting symptoms, management and outcomes of CVD (4). For instance, Lee reported that women with diabetes have a significantly higher CVD mortality rate than men with diabetes (5). Women of reproductive age present with cyclic endocrine changes that might result in different MetS and CVD risk factors in comparison with men. Menstrual cycle irregularity may be a marker of metabolic abnormalities predisposing women to an increased risk for CVD (6). The most well-known correlation between metabolic syndrome and reproductive disorders is in women with polycystic ovary syndrome (PCOS), which is diagnosed by hyperandrogenism and chronic anovulation. Although studies of PCOS and metabolic complications have been widely reported, the understanding of the correlation between endocrine status and metabolic complications in reproductive aged women remains limited and controversial (7-10).

Definitions of metabolic syndrome are usually problematic because they are based on arbitrary cutoff points for several quantitative variables, where each variable is related linearly to cardiovascular risk (11). Further, the risks of developing MetS and CVD vary depending on race and gender. To understand the risk factors of cardiovascular and metabolic disease in reproductive aged women, the studied subjects should be

specified. Cluster analysis is a statistical method based on algorithms that aims to minimize within-group variation and maximize between-group variation for the clustering variables (11). This technique is suitable for defining groups and reflecting the natural structure of data without relying on inappropriate arbitrary cutoffs (12). Cluster analysis can be used to identify groups of women sharing similar metabolic risk factor patterns. We conducted this retrospective study on reproductive age Taiwanese women, and we used cluster analysis to investigate the relationship between metabolic complications and biochemical/clinical characteristics of endocrinologic dysfunction in women of reproductive age.

Materials and Methods

This study was approved by the Institutional Review Board of Taipei Medical University – Wan Fang Hospital, Taipei, Taiwan with the identifier Hsu2013-TMU-JIRB 201302002 and registered at ClinicalTrials.gov with the identifier NCT01826357. We retrospectively reviewed the medical records of female patients who visited our Reproductive Endocrinology Clinic from Jan. 1, 2009, to Jun. 31, 2012.

Parameters of cardiovascular risk

Metabolic syndrome is a complex of interrelated risk factors for CVD and diabetes. These factors include dysglycemia, high blood pressure, elevated triglyceride levels, low high-density lipoprotein cholesterol levels, and central adiposity (1). To evaluate the risk of MetS and CVD, the following ten cardio-metabolic parameters were used for initial cluster analysis in this study: systolic blood pressure, diastolic blood pressure, waist size, fasting insulin, fasting glucose, 2-hour glucose, total cholesterol, triglyceride, high-density lipoprotein (HDL), and low-density lipoprotein (LDL).

Study data

Women who had a complete set of anthropometric measurements and clinical and biochemical data about endocrinologic and cardiovascular parameters were initially included. The chief complaints of studied patients were menstrual irregularity, infertility, overweight, acne/hirsutism, healthy volunteer, and others (more than one complains, transferred from other medical specialists, headache, abdomen pain, vaginal itching, etc.). Subjects' medical histories included a detailed menstrual and medical/surgical history, anthropometric measurements (weight, height, waist, and hip), and blood pressure. The dates and assays performed for blood sampling have been previously described (13). The following data were collected and calculated: (1)

serum androgens, including total testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEA-S), 17-α-OH progesterone, and free androgen index (FAI); (2) insulin sensitivity and glucose tolerance, including fasting insulin, fasting glucose, two-hour glucose, and the homeostasis model assessment insulin resistance index (HOMA-IR); (3) lipid profiles, including total cholesterol, triglycerides, HDL, and LDL; (4) liver function and inflammatory markers, including glutamic oxaloacetic transaminase(GOT), glutamic pyruvic transaminase (GPT), and high sensitivity C-reactive protein (hs-CRP); and (5) sex hormone-binding globulin (SHBG), prolactin, and anti-Müllerian hormone (AMH).

The risks of metabolic syndrome, impaired glucose tolerance and diabetes were evaluated in every studied subject. The waist-to-hip ratio (WHR) was defined as the waist circumference/hip circumference. Body mass index (BMI) was defined as the body weight in kilograms divided by the body height in meters squared (kg/m^2). Overweight/obesity was defined as BMI $\geq$ 25 kg/m^2. All studied women received an ultrasonography examination. Although vaginal ultrasound examination is preferred for young women without sexual experience, abdominal ultrasounds were performed to detect polycystic ovaries..

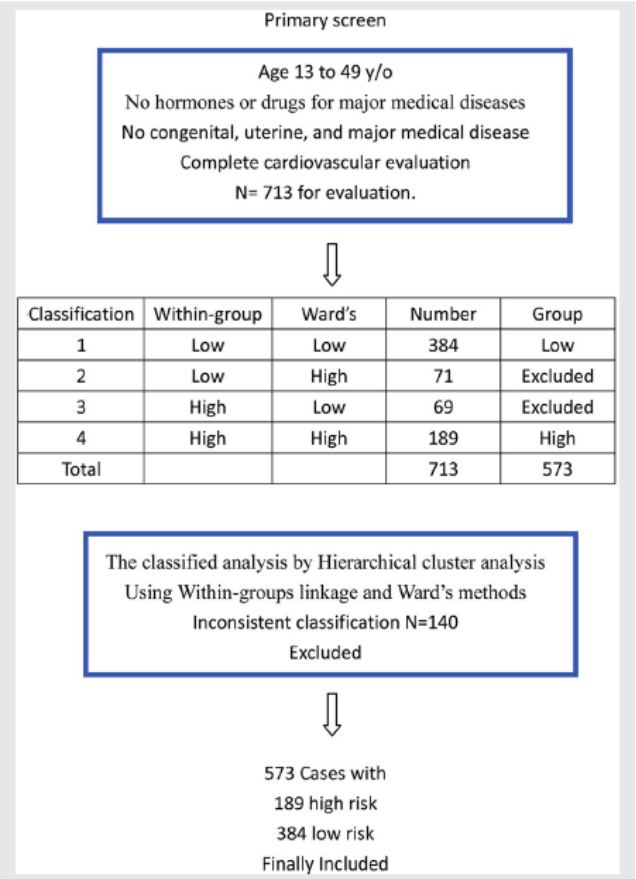
Premature ovarian failure (POF) was defined as oligo/amenorrhea in women under 40 years of age with elevated serum FSH levels (FSH > 16 mIU/mL). A diagnosis of POF was confirmed by serum examination of FSH two weeks later. Hyperprolactinemia was diagnosed when prolactin levels were above the upper limit of normal (24.20 ng/mL).

The following women were excluded from the study populations: (1) women who had been diagnosed with malignant tumors, Asherman's syndrome, Mullerian agenesis, or chromosomal anomalies; (2) women who had menarche within the past 1 year or were older than 49 years old; and (3) women who received hormones, and medicines for diabetes, hypertension, dyslipidemia within the previous three months.
A total of 713 women were included in the study for evaluation. To perform the cluster analysis, the above-mentioned 10 parameters were used to evaluate the risks of developing MetS and CVD (systolic pressure, diastolic pressure, waist size, fasting glucose, fasting insulin, 2-hour glucose, total cholesterol, triglyceride, HDL, and LDL).

To identify the clinical and biochemical characteristics in the high-risk MetS and CVD group, the studied patients were classified into two comparative groups. The classification of the high- and low-risk groups was performed by hierarchical cluster analysis using both within-group linkage and Ward's method. With the within-group linkage method, 258 cases were classified as high-risk patients, and 455 were classified as low-risk patients. However, with Ward's analysis, 260 and 453 patients

were classified as high risk and low risk, respectively. To verify the data, 189 and 384 patients who were confirmed to be in the high-risk and low-risk groups, respectively, with both methods were used for the final analysis, and 140 cases were further excluded due to inconsistent classification with the two different methods (Figure 1). Women with missing endocrinologic or metabolic data were excluded. All included 573 cases featured complete data for all parameters.

FIGURE 1



Classification	Within-group	Ward's	Number	Group
1	Low	Low	384	Low
2	Low	High	71	Excluded
3	High	Low	69	Excluded
4	High	High	189	High
Total			713	573

Flow chart of patient selection in this study.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

PCOS was diagnosed according to the Androgen Excess and PCOS Society criteria (14), which require the presence of hyperandrogenism (hirsutism and/or biochemical) and ovarian dysfunction (oligo-anovulation and /or polycystic ovaries). The definitions of oligo-anovulation and polycystic ovaries have been described in detail previously (15). Hyperandrogenism (HA) was defined as hirsutism and/or biochemical hyperandrogenemia (BioHA). Biochemical hyperandrogenemia was defined as a total serum testosterone value ≥ 0.8 ng/mL (normal range for female adults is 0.1-0.8 ng/mL), androstenedione ≥ 2.99 ng/dL (normal range for females is 0.10-2.99 ng/mL), or DHEAS ≥ 275 μg/L (16). Hirsutism was defined as a modified Ferriman–Gallwey (mF-G) score of ≥6.

The insulin sensitivity index was evaluated by the homeostasis model assessment insulin resistance index (HOMA-IR) using the following formula:

HOMA-IR = [Fasting insulin (μU/mL) × Fasting glucose (mg/dL)]/405

Impaired glucose tolerance (IGT) was defined as two-hour glucose levels of 140 to 199 mg/dL in the 75-g oral glucose tolerance test. In women with IGT, the fasting plasma glucose (FPG) level should be less than 126 mg/dl.

Metabolic syndrome (MBS) was defined (2005 National Cholesterol Education Program - Adult Treatment Panel III) as the presence of at least three of the following criteria: abdominal obesity (waist circumference > 80 cm in women), serum triglycerides ≥ 150 mg/dL, serum HDL<50 mg/dL, systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, and fasting plasma glucose ≥100 mg/dL.

Statistical analysis

We performed cluster analysis to identify groups of women with similar cardiometabolic risk factor patterns using two-step cluster analysis with Statistical Package for Social Science 15.0 software (SPSS Institute Inc., Chicago, Illinois, USA). The number of clusters is determined automatically.

In Tables 1 and 2, data are presented as the mean ± standard deviation. We used Chi-square and Fisher's exact tests to perform categorical comparisons and ANOVA to compare the continuous variables. The means of more than two groups were compared using one-way ANOVA post hoc range (Dunnett's) tests with equal variances not assumed. The differences between the groups were considered significant if the P-values were less than 0.05.

Results

Among the 573 studied patients, 384 and 189 patients were classified as being low and high risk, respectively. For clinical diagnosis, 235 (47%) subjects had polycystic ovarian syndrome, 55 (10%) had hyperprolactinemia, and 17 (3%) had premature ovarian failure. In terms of metabolic complications, 128 (22%) had metabolic syndrome, 48 (9%) had impaired glucose tolerance, and 13 (2%) had diabetes mellitus.

Table 1 shows the ten parameters related to cardiovascular and metabolic risk in the high- and low-risk groups. Nine out of ten parameters (all except serum total cholesterol level) were significantly different between the two groups ($p<0.001$). The results show that the above classifications can be used to separate high- and low-risk patients in terms of cardiovascular and metabolic risk.

TABLE 1

Parameters of cardiovascular and metabolic risk in high-risk and low-risk women.

Parameter	Total	Low risk	High risk	*P* value
Case number	573	384	189	
Systolic pressure (mm Hg)	112.0 ± 17.1	104.9 ± 12.2	126.4 ± 16.4	<.001[a]
Diastolic pressure (mm Hg)	75.9 ± 13.0	70.7 ± 9.4	86.5 ± 13.0	<.001[a]
Waist (cm)	82.1 ± 15.3	73.4 ± 7.3	99.8 ± 11.6	<.001[a]
Fasting insulin (μIU/mL)	12.5 ± 12.1	8.0 ± 4.3	21.7 ± 16.8	<.001[a]
Fasting glucose (mg/dL)	90.2 ± 9.5	87.4 ± 6.6	95.8 ± 11.6	<.001[a]
2-hour glucose (mg/dL)	108.1 ± 32.6	96.3 ± 20.9	132.1 ± 38.4	<.001[a]
Cholesterol (mg/dL)	183.7 ± 32.1	185.0 ± 31.9	181.0 ± 32.3	.160
Triglycerides (mg/dL)	81.4 ± 68.7	60.6 ± 26.9	123.5 ± 101.0	<.001[a]
High-density lipoprotein (mg/dL)	56.2 ± 16.6	64.0 ± 14.3	40.4 ± 6.7	<.001[a]
Low-density lipoprotein (mg/dL)	107.1 ± 27.9	101.7 ± 26.7	118.0 ± 27.0	<.001[a]

Note: Data are mean ± standard deviation.
[a] $P < .05$.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

Table 2 presents the clinical and biochemical characteristics of patients at high and low risk of cardiovascular and metabolic diseases. All parameters related to metabolic risk and insulin resistance were significantly different between the high- and low-risk groups. Patients with hyperprolactinemia and premature ovarian failure did not exhibit an increased cardiovascular and metabolic risk. In contrast, the high-risk group had higher prevalences of hyperandrogenism, oligomenorrhea, polycystic ovary morphology, and PCOS. In terms of androgens, the high-risk group had a higher serum total testosterone and free androgen index than the low-risk group; however, the serum androstenedione and DHEAS levels did not differ between the high- and low-risk groups.

TABLE 2

Clinical and biochemical characteristics of women at high and low risk of cardiovascular and metabolic disorder.

Characteristic	Total	Low risk	High risk	*P* value
Case no.	573	384	189	
Age (y)	27.3 ± 6.5	26.9 ± 6.4	27.9 ± 6.6	.096
Menarche (y)	12.6 ± 1.5	12.7 ± 1.5	12.3 ± 1.4	.001[a]
hs-CRP[a] (mg/L)	0.22 ± 0.38	0.12 ± 0.27	0.44 ± 0.46	<.001[a]
SHBG (ng/dL)	45.8 ± 29.3	56.2 ± 29.1	24.9 ± 15.1	<.001[a]
Antimüllerian hormone (ng/mL)	7.46 ± 5.44	7.67 ± 5.47	7.02 ± 5.36	.175
Anthropometric measurements				
Weight (kg)	63.3 ± 17.4	53.4 ± 7.5	83.3 ± 14.4	<.001[a]
Height (cm)	160.5 ± 5.3	160.0 ± 5.1	161.3 ± 5.6	.008[a]
BMI (kg/m^2)	24.5 ± 6.5	20.8 ± 2.6	32.0 ± 5.3	<.001[a]
Hip (cm)	98.7 ± 11.6	92.6 ± 6.6	111.0 ± 9.7	<.001[a]
Waist to hip ratio	0.83 ± 0.09	0.79 ± 0.07	0.90 ± 0.07	<.001[a]
Hyperprolactinemia	10%	11%	7%	.121
POF	3%	4%	2%	.173
PCOS	47%	39%	61%	<.001[a]
Oligo/amenorrhea	56%	51%	64%	.003[a]
Polycystic ovary morphology	57%	54%	64%	.018[a]
Hyperandrogenism	54%	47%	67%	<.001[a]
Biochemical hyperandrogenemia	36%	30%	47%	<.001[a]
Hirsutism	34%	29%	43%	.001[a]
Metabolism				
Metabolic syndrome	22%	1%	66%	<.001[a]
Hypertension	27%	10%	62%	<.001[a]
HDL <50 mg/dL	39%	14%	92%	<.001[a]
Triglycerides >150 mg/dL	10%	2%	26%	<.001[a]
Waist >80 cm	45%	18%	98%	<.001[a]
Impaired glucose tolerance	9%	2%	22%	<.001[a]
Diabetes mellitus	2%	0	7%	<.001[a]
Androgens				
Total testosterone (ng/mL)	0.60 ± 0.29	0.54 ± 0.26	0.72 ± 0.33	<.001[a]
Androstenedione (ng/dL)	2.71 ± 1.37	2.66 ± 1.36	2.79 ± 1.40	.301
Free androgen index	7.68 ± 8.30	4.59 ± 3.78	13.9 ± 11.0	<.001[a]
DHEAS (ng/dL)	197.3 ± 103.4	193.9 ± 97.9	204.1 ± 113.7	.271
17-OH PRG (ng/dL)	1.14 ± 0.99	1.20 ± 1.11	1.02 ± 0.69	.041[a]
mFG score	5.80 ± 4.73	5.27 ± 4.18	6.90 ± 5.54	<.001[a]
Insulin sensitivity and liver function				
HOMA-IR	2.92 ± 3.25	1.75 ± 0.97	5.29 ± 4.67	<.001[a]
GOT IU/L	23.4 ± 11.6	20.6 ± 6.5	29.0 ± 06.6	<.001[a]
GPT IU/L	23.1 ± 19.7	17.0 ± 9.2	35.6 ± 27.8	<.001[a]

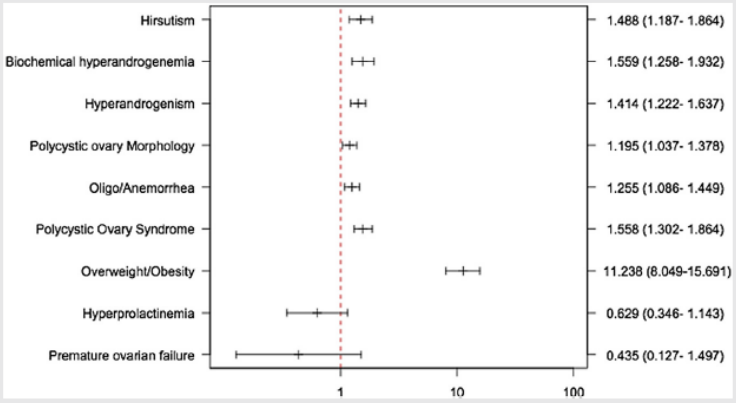
Note: Data are either mean ± SD or are percentage. BMI body mass index; DHEA S dehydroepiandrosterone sulfate; GOT glutamic oxaloacetic transaminase; GPT glutamic pyruvic transaminase; HDL high density lipoprotein; HOMA IR The homeostasis model assessment insulin resistance index; hs CRP high sensitivity C reactive protein; modified mFG Score Ferriman Gallwey score; 17 OH PRG 17 α OH progesterone; PCOS polycystic ovary syndrome; POF premature ovarian failure; SHBG sex hormone binding globulin.

[a] *P*< .05.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

Figure 2 shows the odds ratios for subjects at high risk for cardiovascular and metabolic disease. Overweight/obesity (odds ratio: 11.2, 95% confidence interval: 8.0-15.7), PCOS (1.6; 1.3-1.9), oligo/amenorrhea (1.3, 1.1-1.4), polycystic ovary morphology (1.2, 1.0-1.4), hyperandrogenism (1.4, 1.2-1.6), and biochemical hyperandrogenemia (1.6, 1.3-1.9). Hyperprolactinemia (0.6, 0.3-1.1) and premature ovarian failure (0.4, 0.1-1.5) were not associated with an increased risk of cardiovascular and metabolic diseases.

FIGURE 2



Odds ratio for the high risk of a clinical diagnosis of cardiovascular disease.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

Discussion

Metabolic syndrome (MetS) is a cluster of cardio-metabolic factors that predisposes individuals to diabetes and cardiovascular disease (CVD). The question of whether MetS is a simple aggregation of associated metabolic risk factors or a true syndrome arising from a common physiological origin is controversial (17). Even congruent definitions differ between international authorities and continue to change (18). Cutoff values for risk factors should be determined, and these definitions should reflect gender-based and ethnic differences. To avoid the bias of gender and ethnic variance, our study included reproductive aged Taiwanese women only. Instead of using arbitrary cutoff values, we performed cluster analysis using ten cardiovascular and metabolic risk parameters and classified the study population into high- and low-risk groups. Cluster analysis was a useful tool for identifying groups of women sharing similar metabolic risk factor patterns. The main purpose of this study was to identify the clinical and biochemical characteristics of high and low metabolic risk in reproductive women. To confirm the classification of the high- and low-risk groups, two cluster analysis methods were applied and confirmed in our study. We excluded 140 patients who could not be consistently classified by the two statistical methods. Finally, the parameters related to cardiovascular disease, metabolic syndrome, and insulin resistance were significantly different between the high- and low-risk groups. Metabolic syndrome was diagnosed by the cluster of abdominal obesity, hyperglycemia, hypertension, and dyslipidemia, which increases the risk for type 2 diabetes and cardiovascular diseases (19). We believe this classification could separate the high and low MetS and CVD risk groups in our studied populations.

The metabolic profile noted in women with PCOS is similar to that of insulin

resistance syndrome and consists of hyperinsulinemia, mild glucose intolerance, dyslipidemia, and hypertension (20). Nine out of ten parameters, all except total cholesterol, that are related to MetS and CVD risk (blood pressure, waist size, insulin, glucose, triglyceride, HDL, and LDL) were strongly associated with the high-risk group in our study. The association between PCOS and metabolic disturbance has been widely reported. The risk of metabolic syndrome may vary among the four phenotypes of PCOS based on the Rotterdam criteria (9). Hyperinsulinemia was reported to correlate with free testosterone levels only in women with traditional NIH-defined PCOS (7). Goverde reported that hyperandrogenic PCOS phenotypes are strongly linked to metabolic syndrome and insulin resistance in Dutch women with PCOS (21). Legro reported that neither the morphology nor the volume of the ovaries is associated with distinctive metabolic or reproductive phenotypes in women with PCOS (8). In most patients, PCOS-related disturbances (hyperandrogenism, ovulatory dysfunction, and polycystic ovary morphology) may cluster together, making it difficult to evaluate them individually. Our study shows that all PCOS-related disorders, such as ovulatory dysfunction, hyperandrogenism, hirsutism and polycystic ovary morphology, clustered together and were strongly associated with high risk of MetS and CVD. Furthermore, the high-risk group had higher levels of inflammatory markers and liver enzymes and lower SHBG than the low-risk group. Interestingly, the high-risk group had an earlier menarche than the low-risk group. We found that hyperprolactinemia and premature ovarian failure were not related with MetS and CVD risk. Androgen levels are the major distinguishing endocrine feature differentiating the phenotypic expressions of PCOS. Androgen excess in women may signal a risk for coronary artery disease (20). However, the different types of androgens should be evaluated separately. Specifically, the serum total testosterone level and free androgen index, but not androstenedione or DHEAS, were associated with the risk of MetS and CVD.

Obesity should be the major impact factor for reproductive women with a high risk of MetS and CVD. The association between obesity and a cluster of cardiometabolic risk factors is stronger in women than in men, and this gender-specific difference exists in younger but not in older individuals (22). Obesity accounts for the maximum variance in clustering and appears to be a more powerful correlate of cardiovascular risk in children and adolescents (23). Ketel, using a isoglycemic-hyperinsulinemic clamp, showed that PCOS per se was not associated with impaired metabolic insulin sensitivity in normal-weight women, but it aggravates the impairment of metabolic insulin sensitivity in obese women (24). Spranger measured the insulin resistance of women with PCOS by the continuous infusion of glucose and model assessment and demonstrated that BMI but not testosterone was independently associated with insulin

sensitivity (25). Similarly, in our study, obesity was the major factor determining the risks of MetS and CVD in reproductive aged women. Although PCOS and obesity both increased the risks of MetS and CVD, the odds ratio in high-risk women with PCOS (1.6, 95% confidence interval 1.3-1.9) was much lower than that for women who were overweight/obese (11.2, 95% 8.0-15.7).

The major weakness of this study is that the women evaluated in the present study were recruited from the outpatient clinic of a tertiary care center and do not reflect the true distribution of the general population. Therefore, the results should be applied to the general population with caution.

Finally, we can summarize our studied results with the following points:

1. Overweight/obesity was the major determinant of cardiovascular and metabolic disturbances in reproductive aged women.
2. Early menarche, high levels of inflammatory markers and liver enzymes, and low SHBG were associated with high cardiovascular and metabolic risk.
3. Hyperprolactinemia and premature ovarian failure were not associated with cardiovascular and metabolic risk.
4. Oligomenorrhea, hyperandrogenism, and PCOS were associated with high cardiovascular and metabolic risk.
5. The serum total testosterone level and free androgen index, but not androstenedione or DHEAS, were associated with cardiovascular and metabolic risk.

Reference

1. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009;120:1640-5.
2. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet. 2005;365:1415-28.
3. Taverna MJ, Martínez-Larrad MT, Frechtel GD, Serrano-Ríos M. Lipid accumulation product: a powerful marker of metabolic syndrome in healthy population. Eur J Endocrinol. 2011;164:559-67.
4. Pilote L, Dasgupta K, Guru V, Humphries KH, McGrath J, Norris C, et al. A comprehensive view of sex-specific issues related to cardiovascular disease. CMAJ. 2007;176:S1-44.

5. Lee WL, Cheung AM, Cape D, Zinman B. Impact of diabetes on coronary artery disease in women and men: a meta-analysis of prospective studies. Diabetes Care. 2000;23:962-8.
6. Solomon CG, Hu FB, Dunaif A, Rich-Edwards JE, Stampfer MJ, Willett WC, et al. Menstrual cycle irregularity and risk for future cardiovascular disease. J Clin Endocrinol Metab. 2002;87:2013-7.
7. Kauffman RP, Baker TE, Baker VM, DiMarino P, Castracane VD. Endocrine and metabolic differences among phenotypic expressions of polycystic ovary syndrome according to the 2003 Rotterdam consensus criteria. Am J Obstet Gynecol. 2008;98:670.e1-7.
8. Legro RS, Chiu P, Kunselman AR, Bentley CM, Dodson WC, Dunaif A. Polycystic ovaries are common in women with hyperandrogenic chronic anovulation but do not predict metabolic or reproductive phenotype. J Clin Endocrinol Metab. 2005;90:2571-9
9. Shroff R, Syrop CH, Davis W, Van Voorhis BJ, Dokras A. Risk of metabolic complications in the new PCOS phenotypes based on the Rotterdam criteria. Fertil Steril. 2007;88:1389-95.
10. Welt CK, Gudmundsson JA, Arason G, Adams J, Palsdottir H, Gudlaugsdottir G, et al. Characterizing discrete subsets of polycystic ovary syndrome as defined by the Rotterdam criteria: the impact of weight on phenotype and metabolic features. J Clin Endocrinol Metab. 2006;91:4842-8.
11. Huang RC, Burke V, Newnham JP, Stanley FJ, Kendall GE, Landau LI, et al. Perinatal and childhood origins of cardiovascular disease. Int J Obes (Lond). 2007;31:236-44.
12. Hart R, Doherty DA, Mori T, Huang RC, Norman RJ, Franks S, et al. Extent of metabolic risk in adolescent girls with features of polycystic ovary syndrome. Fertil Steril. 2011;95:2347-53, 2353.e1.
13. Liang SJ, Hsu CS, Tzeng CR, Chen CH, Hsu MI. Clinical and biochemical presentation of polycystic ovary syndrome in women between the ages of 20 and 40. Hum Reprod. 2011;26:3443-9.
14. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al; Task Force on the Phenotype of the Polycystic Ovary Syndrome of The Androgen Excess and PCOS Society. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. Fertil Steril. 2009;91:456-88.
15. Hsu MI, Liou TH, Liang SJ, Su HW, Wu CH, Hsu CS. Inappropriate gonadotropin secretion in polycystic ovary syndrome. Fertil Steril. 2009;91:1168-74.

16. Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab. 2004;89:2745-9.
17. Yoon JH, Park JK, Oh SS, Lee KH, Kim SK, Kim JK, et al. The clustering patterns of metabolic risk factors and its association with sub-clinical atherosclerosis in Korean population. Ann Hum Biol. 2011;38:640-6.
18. Jørgensen ME, Bjerregaard P, Gyntelberg F, Borch-Johnsen K; Greenland Population Study. Prevalence of the metabolic syndrome among the Inuit in Greenland. A comparison between two proposed definitions. Diabet Med. 2004;21:1237-42.
19. Povel CM, Beulens JW, van der Schouw YT, Dollé ME, Spijkerman AM, Verschuren WM, et al. Metabolic Syndrome Model Definitions Predicting Type 2 Diabetes and Cardiovascular Disease. Diabetes Care. 2013;36:362-8.
20. Wild RA, Grubb B, Hartz A, Van Nort JJ, Bachman W, Bartholomew M. Clinical signs of androgen excess as risk factors for coronary artery disease. Fertil Steril. 1990;54:255-9.
21. Goverde AJ, van Koert AJ, Eijkemans MJ, Knauff EA, Westerveld HE, Fauser BC, et al. Indicators for metabolic disturbances in anovulatory women with polycystic ovary syndrome diagnosed according to the Rotterdam consensus criteria. Hum Reprod. 2009;24:710-7.
22. Wakabayashi I. Age-dependent influence of gender on the association between obesity and a cluster of cardiometabolic risk factors. Gend Med. 2012;9:267-77.
23. Khader YS, Batieha A, Jaddou H, Batieha Z, El-Khateeb M, Ajlouni K. Factor analysis of cardiometabolic risk factors clustering in children and adolescents. Metab Syndr Relat Disord. 2011;9:151-6.
24. Ketel IJ, Stehouwer CD, Serné EH, Korsen TJ, Hompes PG, Smulders YM, et al. Obese but not normal-weight women with polycystic ovary syndrome are characterized by metabolic and microvascular insulin resistance. J Clin Endocrinol Metab. 2008;93:3365-72.
25. Spranger J, Möhlig M, Wegewitz U, Ristow M, Pfeiffer AF, Schill T, et al. Adiponectin is independently associated with insulin sensitivity in women with polycystic ovary syndrome. Clin Endocrinol (Oxf). 2004;61:738-46.

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否有嚴重損及公共利益之發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■ 達成目標

□ 未達成目標（請說明，以 100 字為限）

□ 實驗失敗

□ 因故實驗中斷

□ 其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性），如已有嚴重損及公共利益之發現，請簡述可能損及之相關程度（以 500 字為限）

本研究利用集群方法分析數據資料，將納入的 573 位女性分成具低風險(384 人)和高風險(159 人)心血管疾病兩組，探討心血管疾病和代謝症候群之間的關係。研究結果發現過重或肥胖、寡經或無經症以及高雄性激素症會提高心血管疾病和代謝疾病之風險，而目前的研究中已知女性常見的多囊性卵巢症候群與代謝症候群相關，但肥胖是生育年齡婦女造成心血管疾病和代謝症候群最主要的原因。因此，我們希望藉由女性內分泌檢查和代謝相關指標來尋找對女性適當的醫療照護。

其他資料

檢附相關論文

1. Ko PC, Huang SY, Hsieh CH, Hsu MI, Hsu CS Serum Ferritin levels and polycystic ovary syndrome in obese and non-obese women. Taiwan J Obstet Gynecol. 2014
2. Chen CI, Hsu MI, Lin SH, Chang YC Ivan, Hsu CS, Tzeng CR. Obesity biomarkers in obese and non-obese women with polycystic ovary syndrome.
3. Lee CT, Wang JY, Chou KY, Hsu MI. 1,25-dihydroxyvitamin D3 increases testosterone-induced 17beta-estradiol secretion and reverses testosterone-reducedconnexin 43 in rat granulosa cells. Reprod Biol Endocrinol. 2014;12:90
4. Tzeng CR, Chang YC, Chang YC, Wang CW, Chen CH, Hsu MI. Cluster analysis of cardiovascular and metabolic risk factors in women of reproductive age. Fertil Steril. 2014;101(5):1404-10

台灣婦產科醫學會

會刊雜誌受刊證明

作　　者：Po-Chun KO, Shih-Yi HUANG, Ching-Hung HSIEH, Ming-I HSU, Chun-Sen HSU.

通訊作者：Dr. Ming-I Hsu

投稿編號：2013-403

所著論文：Serum ferritin levels and polycystic ovary syndrome in obese and non-obese women.

已通過本學會雜誌編輯審查會，決議受予刊登於「Taiwanese Journal of Obstetrics and Gynecology（台灣婦產科醫學會會刊雜誌）」。

特此證明

台灣婦產科醫學會　雜誌編輯委員會

2014 年 6 月 4 日

Serum ferritin levels and polycystic ovary syndrome in obese and non-obese women

Po-Chun KO M.D.[1], Shih-Yi HUANG Ph.D.[2], Ching-Hung HSIEH M.D. Ph.D.[3], Ming-I HSU MD[1], Chun-Sen HSU MD[1].

[1]Department of Obstetrics and Gynaecology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan

[2]School of Nutrition and Health Sciences, College of Public Health and Nutrition, Taipei Medical University, Taipei, Taiwan

[3]Department of Obstetrics and Gynecology, Clinic of Fu Jen Catholic University, Taipei, Taiwan

PC KO and SY HUANG contributed equally to this work

MI HSU and CS HSU contributed equally to this work

The authors report no conflict of interest

An abstract describing this work was submitted to 5th International Congress on Prediabetes and the Metabolic Syndrome, Vienna, Austria, April 18-20, 2013

This work was supported by a National Science Council Grant NSC 102-2629-B-038-001) and a Taipei Medical University – Wan Fang Hospital Grant (101TMU-WFH-03).

Reprint requests:

Ming-I Hsu, M.D.

Department of Obstetrics and Gynaecology, Wan Fang Hospital, Taipei Medical University

No.111, Sec. 3, Xinglong Rd., Taipei 11696, Taiwan

Tel: +886-2-29307930

Fax: +886-2-29300036

E-mail: hsumingi@yahoo.com.tw

1 Running title

2 Ferritin andobesity

OBJECTIVE - This study is aimed to evaluate serum ferritin levels and polycystic ovary syndrome (PCOS) – related complications in obese and non-obese women.

MATERIALS AND METHODS –This retrospective study included 539 (286 with PCOS and 253 without PCOS).

RESULTS -Serum ferritin correlated with menstrual cycle length, sex hormone-binding globulin, total testosterone, androstenedione, triglyceride, and total cholesterol in both obese and non-obese women. Obese women with high ferritin levels exhibited higher insulin resistance, impaired glucose tolerance, and liver enzymes (GOT, GPT) than obese women with low ferritin levels. However, among non-obese women, insulin resistance and risk of diabetes were not significantly different between the high and low ferritin groups. Independent of obesity, hypertriglyceridemia was the major metabolic disturbance observed in women with elevated serum ferritin levels.

CONCLUSIONS -Elevated serum ferritin levels are associated with increased insulin resistance and risk of diabetes in obese women but not in non-obese women. However, higher serum ferritin levels were correlated with a greater risk of hyperglycerdemia in both obese and non-obese women. Therefore, hypertriglyceridemia in women with

PCOS might be associated with iron metabolism.

Keywords: polycystic ovary syndrome, ferritin, obesity, insulin resistance, metabolic syndrome

Introduction

Ferritin is a ubiquitous intracellular protein that is essential for the regulation of iron homeostasis. The serum ferritin level is widely used as a clinical biomarker to estimate body iron status. Iron is a strong pro-oxidant, and high body iron levels are associated with an increased level of oxidative stress, which may elevate the risk of type 2 diabetes (1). Mildly elevated body iron stores are associated with statistically significant increases in glucose homeostasis indices (2, 3). Furthermore, patients with elevated iron stores present both insulin resistance and metabolic alterations that put them at increased risk for cardiovascular disease (CVD) (4, 5).

Polycystic ovary syndrome (PCOS) is an endocrine disorder that affects 6 to 7% of premenopausal women (6). PCOS is clinically diagnosed by hyperandrogenism and chronic anovulation; however, its morbidity includes insulin resistance, type 2 diabetes mellitus, hypertension, cardiovascular disease, and infertility (7). Increased serum ferritin levels are frequently observed in women with PCOS (8). An excess of androgen and menstrual dysfunction are correlated with ferritin levels in premenopausal women (9). Factors contributing to potential iron overload in women

with PCOS include the iron-sparing effect of chronic menstrual dysfunction, insulin resistance, and a decrease in hepcidin, which leads to increased iron absorption (10).

Serum ferritin concentrations differ significantly according to sex, body status and ethnicity (3, 11, 12). A growing number of studies suggest a potential link between obesity and altered iron metabolism (11). Furthermore, the association between serum ferritin levels and certain diagnostic components of metabolic syndrome might be different in men and women (12). Menstruating women are at risk for iron deficiency; however, obese menstruating women are at low risk of depleting their iron stores (13). Recently, we reported that obesity is the main factor associated with the prevalence of insulin resistance, impaired glucose tolerance and metabolic syndrome in women with PCOS (14, 15). The correlation between serum ferritin levels and metabolic components in obese and non-obese women is not well-understood. Therefore, we conducted this retrospective study to evaluate the relationship between ferritin levels, insulin resistance, metabolic disturbances, and PCOS-related syndrome among obese and non-obese women.

Materials and Methods

This study was approved by the Taipei Medical University Joint Institutional Review Board (Taipei, Taiwan),and registered in the Protocol Registration System of ClinicalTrials.gov(identifier NCT01600833). The authors report no conflicts of interest.

We retrospectively reviewed the medical records of female patients who visited our clinic from Jan. 1, 2008 to Nov. 30, 2011. The chief complaints of these patients included menstrual disturbance, dysmenorrhea, infertility, and acne/hirsutism. The following subjects were excluded: (1) women who had been diagnosed with congenital adrenal hyperplasia, androgen-secreting tumour, Cushing's syndrome, or disorders of the uterus; (2) women who experienced menarche less than three years before the evaluation or those who were older than 46; and (3)women who received hormones or drugs for major medical diseases. A total of 639 women were initially screened. One hundred women were excluded due to hyperprolactinemia (N=62), ovarian failure (N=18), and insufficient data (N=20). Overall, 539 women were included in this study.

Medical histories included detailed menstrual and medical/surgical records as well as anthropometric measurements. Biochemical hyperandrogenemia was defined as a total serum testosterone ≥ 0.8 ng/mL (normal ranges for female adult 0.1-0.8 ng/mL), androstenedione (A4) ≥ 2.99 ng/dL (normal ranges for females 0.10-2.99 ng/mL), or DHEAS ≥ 275 μg/L (16). Hirsutism was defined as a modified Ferriman–Gallwey (mF-G) score of ≥6.The number of menstrual cycles during the previous year was recorded. Menstrual interval was defined as 365 divided by the number of menstrual cycles in the previous year. Oligomenorrhea/amenorrhea was defined as a menstrual interval of >35 days or fewer than 10menstruation cycles in the previous year. Obesity was defined as having a body mass index (BMI) ≥ 25 kg/m^2. The definition of polycystic ovaries was previously described (17).

PCOS was diagnosed according to the Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome (18), which require the presence of hyperandrogenism and ovarian dysfunction.

Serum ferritin levels were obtained in all 539 women, and the median level was

45.5ng/mL. To evaluate further the clinical and biochemical characteristics of women with different levels of serum ferritin, the study cases were classified into the following subgroups: high ferritin group (ferritin ≥ 45.5ng/mL, n= 270) and low ferritin group (ferritin <45.5ng/mL, n= 269).

Metabolic syndrome (MBS; 2005 National Cholesterol Education Program -Adult Treatment Panel III [ATP III]) was defined as the presence of at least three of the following criteria: abdominal obesity, hypertriglyceridemia (triglycerides ≥150 mg/dL), serum HDL<50, hypertension, and fasting plasma glucose ≥ 100 mg/dL.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL). We evaluated the correlation between serum ferritin levels and related parameters with Pearson's correlation coefficients using the two-tailed method (Table 1). In Table 2, data are presented as the means ± standard deviation. We used the chi-square test and Fisher's exact test to compare categorical variables, and ANOVA was used to compare continuous variables. Differences between the groups were

considered to be significant at $p<0.05$.

RESULTS

Table 1 illustrates the correlation between serum ferritin levels and related parameters in all, obese, and non-obese women. Serum ferritin correlated with menstrual cycle length, sex hormone-binding globulin, total testosterone, androstenedione, triglyceride, and total cholesterol in both obese and non-obese women. Fasting insulin, fasting glucose, HbA1C, HOMA-IR, GOT, and HDL were correlated with serum ferritin levels among obese women, but not among non-obese women. However, a strong correlation between serum ferritin levels and triglycerides was observed in both obese and non-obese women. Obese women had significantly higher serum ferritin (82.1±90.3 vs. 48.8±38.3; $p<0.001$) and hs-CRP (0.39±0.41 vs. 0.13±0.30; $p<0.001$) levels than non-obese women.

Table 2 compares the clinical and biochemical characteristics of women with low and high ferritin level in the obese and non-obese subgroups. Women with high ferritin levels had a greater risk of PCOS and hyperandrogenism than women with low

ferritin levels. Furthermore, women with high ferritin levels had longer menstrual interval, lower SHBG, and higher serum androgens and triglycerides than women with low ferritin levels in both obese and non-obese women. Obese women with high ferritin levels had higher insulin resistance, impaired glucose tolerance, and liver enzymes than obese women with low ferritin levels. However, among non-obese women, insulin resistance and metabolic disturbances were not significantly different between the high and low ferritin groups.

CONCULSIONS

Ferritin is the cellular storage protein for iron, and reduced serum ferritin provides unequivocal evidence of diminished iron stores. In women, a serum ferritin concentration ≥ 150 ng/ml is usually defined as hyperferritinemia (19), but increased serum ferritin concentrations in non-pathological conditions, reflecting subclinical iron overload, are associated with insulin resistance and an increased risk of type 2 diabetes mellitus (3). This observation is likely due to the fact that the normal ranges of serum ferritin are too wide, and the criteria for iron overload are too high (20). To

study the effect of serum ferritin levels in this study, women were classified into high and low serum ferritin groups according to the median ferritin levels in our samples.

Obese women tended to have higher haemoglobin and ferritin concentrations and lower transferrin saturation compared to the non-obese women (11, 21). Previous studies reported that ferritin is elevated in inflammatory conditions, even in the presence of true iron deficiency (22). To consider whether body status may impact the pathological effects of ferritin, we separately analysed obese and non-obese women. Although high serum ferritin was associated with insulin resistance and metabolic disturbance in obese women, we did not observe any association between serum ferritin levels and parameters of insulin resistance in non-obese women. This result could explain why a study of non-obese Korean women determined no significant differences in the HOMA-IR in various serum ferritin levels (23).

Prolonged menstrual cycle length was correlated with higher serum ferritin levels. Factors contributing to potential iron overload might result from reduced menstrual losses, secondary to oligo- or amenorrhea, or from hyperinsulinism, secondary to

insulin resistance, because insulin favours the intestinal absorption and the tissue deposition of iron (10). Regular blood loss during menstruation is a physical characteristic of reproductive-aged women. Serum ferritin concentration is directly related to reticuloendothelial iron stores, and normally, 1 μg/l of serum ferritin corresponds to approximately 8 mg of storage iron (22).Our results indicate that prolonged menstrual cycle length is an important factor for iron overload resulting from reduced menstrual losses in reproductive-aged women. Furthermore, serum ferritin levels and disturbances in insulin resistance were observed in obese women, but not non-obese women. However, the pathogenesis of increased iron stores on insulin resistance among obese and non-obese premenopausal women might be different. Increased iron stores in obese women could be a consequence of insulin resistance (24), whereas reduced menstrual losses in women with oligomenorrhea might be a major factor contributing to increased iron stores in non-obese women.

Independent of obesity, women with higher ferritin levels have a greater risk of hypertriglyceridemia. The mechanism underlying increased serum ferritinin hypertriglyceridemia is not clear. Iron accumulation results from the down-regulation

of the iron-export protein, ferroportin-1, which increases the levels of cytokines, such as tumor necrosis factor–α(TNF–α). TNF–α is involved in hyperferritinemia, and the observed high ferritin concentration could be an inflammatory manifestation. A study by Mateo-Gallego suggested that genetic mechanisms underlying hypertriglyceridemia also favour iron overload (25). Elevated triglyceride level is a predictor of cardiovascular disease (CVD) in women (25-27). The American Heart Association recently issued a scientific statement that enforced the pivotal role of triglycerides in lipid metabolism, reaffirming that triglycerides are not directly atherogenic, but represent an important biomarker of CVD risk (28). In terms of metabolic syndrome, the risk of hypertriglyceridemia was significantly increased in women with high ferritin levels. A previous study reported that elevated serum ferritin levels may be employed as a marker of metabolic syndrome in non-obese women (23); however, the association between serum triglycerides and ferritin might be used to understand metabolic disturbances in non-obese women with elevated ferritin levels.Because serum ferritin levels might be a good indicator of menstrual pattern and metabolic disturbance, measuring serum ferritin levels might be useful for evaluating cardiovascular risk in women of reproductive age.

There are several limitations to our study. Serum ferritin concentrations differ significantly according to sex, body status and ethnicity. Therefore, we classified our population into two subgroups (high and low ferritin groups) based on the median (45.5 ng/mL) of our data. The cut-off points employed in this study to determine higher and lower ferritin levels might not apply to other studies. This is a cross-sectional, retrospective study, and the subjects were Taiwanese patients who visited our reproductive endocrine outpatient department during a fixed interval. The average body weight of Taiwanese women is lower than that of Western women; therefore, our results should be applied to the general population with caution.

Acknowledgments

This work was supported by a National Science Council Grant (NSC 102-2629-B-038-001) and a Taipei Medical University – Wan Fang Hospital Grant (101TMU-WFH-03).

References

1. Rajpathak SN, Crandall JP, Wylie-Rosett J, Kabat GC, Rohan TE, Hu FB. The role of iron in type 2 diabetes in humans. Biochim Biophys Acta. 2009;1790:671-81

2. Tuonmainen TP, Nyyssonen K, Salonen R, Tervahauta A, Korpela H, Lakka T, Kaplan GA, Salonen JT. Body iron stores are associated with serum insulin and blood glucose concentrations: population study in 1,013 eastern Finnish men. Diabetes Care 1997;20:426-8

3. Kim CH, Kim HK, Bae SJ, Park JY, Lee KU. Association of elevated serum ferritin concentration with insulin resistance and impaired glucose metabolism in Korean men and women. Metabolism. 2011;60:414-20

4. Jehn M, Clark JM, Guallar E: Serum ferritin and risk of metabolic syndrome in U.S. adults. Diabetes Care 2004;27:2422-8

5. Meroño T, Rosso LG, Sorroche P, Boero L, Arbelbide J, Brites F. High risk of cardiovascular disease in iron overload patients.Eur J Clin Invest. 2011;41:479-86

6. Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab. 2004;89: 2745-9.

7. Lobo RA, Carmina E. The importance of diagnosing the polycystic ovary syndrome. Ann Intern Med. 2000;132: 989-93.

8. Escobar-Morreale HF, Luque-Ramirez M, Alvarez-Blasco F, Botella-Carretero JI, Sancho J, San Millan JL: Body iron stores are increased in overweight and obese women with polycystic ovary syndrome. Diabetes Care 2005;28:2042-4

9. Martinez-Garcia MA, Luque-Ramirez M, San-Millan JL, Escobar-Morreale HF. Body iron stores and glucose intolerance in premenopausal women. Diabetes Care 2009;32:1525-30

10. Escobar-Morreale HF. Iron metabolism and the polycystic ovary syndrome. Trends EndocrinolMetab. 2012;23:509-15

11. Cheng HL, Bryant C, Cook R, O'Connor H, Rooney K, Steinbeck K. The relationship between obesity and hypoferraemia in adults: a systematic review. Obes Rev. 2012;13:150-61.

12. Kang HT, Linton JA, Shim JY. Serum ferritin level is associated with the prevalence of metabolic syndrome in Korean adults: the 2007-2008 Korean National Health and Nutrition Examination Survey. Clin Chim Acta. 2012;413:636-41.

13. Fricker J, Le Moel G, Apfelbaum M. Obesity and iron status in menstruating women. Am J Clin Nutr. 1990;52:863-6.

14. Chun-Sen H, Chien-Hua W, Wan-Chun C, Ching-Tzu L, Chun-Jen C, Hsu MI. Obesity and insulin resistance in women with polycystic ovary syndrome. Gynecol Endocrinol. 2011; 27:300-6

15. Liang SJ, Liou TH, Lin HW, Hsu CS, Tzeng CR, Hsu MI. Obesity is the predominant predictor of impaired glucose tolerance and metabolic disturbance in polycystic ovary syndrome. Acta Obstet Gynecol Scand. 2012;91:1167-72.

16. Azziz R, Sanchez LA, Knochenhauer ES, Moran C, Lazenby J, Stephens KC, Taylor K, Boots LR. Androgen excess in women: experience with over 1000 consecutive patients. J Clin Endocrinol Metab. 2004 Feb;89(2):453-62.

17. Hsu MI, Liou TH, Liang SJ, Su HW, Wu CH, Hsu CS. Inappropriate gonadotropin secretion in polycystic ovary syndrome. Fertil Steril. 2009 ;91:1168-74.

18. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ, Taylor AE, Witchel SF; Task Force on the Phenotype of the Polycystic Ovary Syndrome of The Androgen Excess and PCOS Society. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. Fertil Steril. 2009;91:456-88.

19. Aso Y, Takebayashi K, Wakabayashi S, Momobayashi A, Sugawara N, Terasawa T, Naruse R, Hara K, Suetsugu M, Morita K, Inukai T. Relation between serum high molecular weight adiponectin and serum ferritin or prohepcidin in patients with type 2 diabetes. Diabetes Res Clin Pract. 2010;90:250-5.

20. Ashourpour M, Djalali M, Djazayery A, Eshraghian MR, Taghdir M, Saedisomeolia A. Relationship between serum ferritin and inflammatory biomarkers with insulin resistance in a Persian population with type 2 diabetes and healthy people. Int J Food Sci Nutr. 2010;61:316-23

21. Yanoff LB, Menzie CM, Denkinger B, Sebring NG, McHugh T, Remaley AT, Yanovski JA. Inflammation and iron deficiency in the hypoferremia of obesity. Int J Obes (Lond). 2007;31:1412-19.

22. Fitzsimons EJ, Brock JH. The anaemia of chronic disease. BMJ. 2001;322:811-2.

23. Yoo KD, Ko SH, Park JE, Ahn YB, Yim HW, Lee WC, Park YM. High serum ferritin levels are associated with metabolic risk factors in non-obese Korean young adults: Korean National Health and Nutrition Examination Survey (KNHANES) IV. Clin Endocrinol (Oxf). 2012;77:233-40

24. Luque-Ramirez M, Alvarez-Blasco F, Botella-Carretero JI, Sanchon R, San Millan JL, Escobar-Morreale HF. The increased body iron stores of obese women with polycystic ovary syndrome are a consequence of insulin resistance and hyperinsulinism and are not a result of reduced menstrual losses. Diabetes Care 2007;30:2309-13

25. Mateo-Gallego R, Calmarza P, Jarauta E, Burillo E, Cenarro A, Civeira F. Serum ferritin is a major determinant of lipid phenotype in familial combined hyperlipidemia and familial hypertriglyceridemia. Metabolism. 2010;59:154-8.

26. Aigner E, Hinz C, Steiner K, Rossmann B, Pfleger J, Hohla F, Steger B, Stadlmayr A, Patsch W, Datz C. Iron stores, liver transaminase levels and metabolic risk in healthy teenagers.Eur J Clin Invest. 2010;40:155-63.

27. Castelli WP. The triglyceride issue: a view from Framingham. Am Heart J. 1986;112:432-7.

28. Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, Goldberg AC, Howard WJ, Jacobson MS, Kris-Etherton PM, Lennie TA, Levi M, Mazzone T, Pennathur S; Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. Circulation. 2011;123:2292-333.

Table legends

Table 1: Correlation of ferritin with clinical and biochemical insulin resistance and metabolic syndrome (n=539).

Table 2: Comparison of the biochemical characteristics of obese and non-obese women with high and low ferritin.

Table 1: Correlation of ferritin with clinical and biochemical insulin resistance and metabolic syndrome (n=539)

	Total (n=539)		obese (n=233)		Non-obese (n=306)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Parameters correlated with ferritin both in obese and non-obese women						
Menstrual cycle length	0.320	<0.001*	0.378	<0.001*	0.124	0.031*
Sex Hormone-binding globulin	-0.249	<0.001*	-0.192	0.003*	-0.197	0.001*
Total testosterone	0.221	<0.001*	0.151	0.021*	0.224	<0.001*
Androstenedione	0.181	<0.001*	0.164	0.012*	0.257	<0.001*
Cholesterol	0.165	<0.001*	0.158	0.016*	0.129	0.026*
Triglyceride	0.334	<0.001*	0.264	<0.001*	0.324	<0.001*
Parameters correlated with ferritin both in obese but not non-obese women						
Fasting insulin	0.257	<0.001*	0.233	0.002*	0.009	0.872
Fasting glucose	0.270	<0.001*	0.258	<0.001*	0.092	0.115
HOMA-IR[a]	0.329	<0.001*	0.306	<0.001*	0.043	0.455
Haemoglobin A1c	0.284	<0.001*	0.258	0.001*	0.068	0.289
GOT[a]	0.561	<0.001*	0.621	<0.001*	0.048	0.411
High-density lipoprotein	-0.196	<0.001*	-0.137	0.037*	-0.045	0.437
Body Mass Index	0.271	<0.001*	0.149	0.023*	0.044	0.442
high-sensitivity C-reactive protein	0.248	<0.001*	0.229	<0.001*	0.074	0.202
Systolic pressure	0.210	<0.001*	0.167	0.013*	-0.049	0.397
Diastolic pressure	0.220	<0.001*	0.175	0.009*	-0.040	0.497

**Note: ***p<0.05

[a] : HOMA-IR : The homeostasis model assessment insulin resistance index, GOT: Glutamic Oxaloacetic Transaminase

Table-2: A comparison of biochemical characteristics of obese and non-obese women with high and low ferritin

	Obese			Non-obese		
	Low Ferritin[a]	High Ferritin[a]	p-value	Low Ferritin[a]	High Ferritin[a]	p-value
Case number	89	144		180	126	
Ferritin (ng/mL)	25.1±12.5	117.4±99.2	<0.001*	25.6±12.1	82.0±38.8	<0.001*
Age (y/o)	29.0±6.7	27.8±6.5	0.180	27.6±6.3	26.5±6.6	0.143
Menstrual cycle length (days)	64.1±64.1	113.4±116.0	<0.001*	61.1±66.5	83.9±81.4	0.008*
BMI (kg/m^2) [a]	31.3±5.2	31.3±4.8	0.951	20.5±2.1	20.6±2.0	0.579
PCOS	44%	62%	0.007*	44%	63%	0.001*
Hyperandrogenism	53%	69%	0.014*	55%	69%	0.013*
Polycystic ovaries	57%	63%	0.433	56%	58%	0.752
Oligo/Amenorrhea	66%	76%	0.094	52%	74%	<0.001*
SHBG (nmol/L) [a]	32.0±17.2	23.0±12.9	<0.001*	58.9±28.8	47.4±26.9	<0.001*
hsCRP (mg/dL) [a]	0.30±0.31	0.47±0.44	0.012*	0.10±0.22	0.17±0.37	0.051
Systolic pressure (mmHg)	120.6±15.5	125.4±18.7	0.051	106.8±13.7	104.9±11.9	0.225
Diastolic pressure (mmHg)	83.6±11.8	87.1±14.2	0.062	71.9±10.0	71.4±9.9	0.688
Anthropometric measurements						
Weight (Kg)	81.8±15.1	81.3±13.0	0.766	52.5±6.0	52.9±6.3	0.496
Height (cm)	161.0±5.3	161.0±5.7	0.444	160.0±4.7	160.0±5.7	0.908
Waist (cm)	96.6±12.6	98.2±12.0	0.331	73.2±6.9	73.8±7.1	0.446
Hip (cm)	111.0±9.1	109.3±8.7	0.154	91.7±5.3	92.0±6.3	0.685
Waist to Hip ratio	0.87±0.08	0.90±0.08	0.007*	0.80±0.08	0.80±0.08	0.591
Androgens						
Total testosterone (ng/mL)	0.64±0.25	0.76±0.33	0.003*	0.51±0.24	0.62±0.30	0.001*
Androstenedione (ng/mL)	2.5±1.3	2.9±1.3	0.017*	2.4±1.1	3.0±1.4	<0.001*
Free Androgen Index [a]	10.1±9.3	15.0±10.2	<0.001*	3.9±3.1	6.2±5.2	<0.001*
DHEAS [a] (ng/dL)	185±82	210±118	0.076	186±99	195±101	0.448
17-OH PRG [a] (ng/dL)	0.9±0.6	1.1±0.7	0.131	1.1±0.9	1.3±1.0	0.283
Insulin sensitivity and glucose tolerance						

Fasting Insulin (uIU/mL)	16.1±11.7	21.9±18.7	0.010*	8.8±10.8	8.5±4.5	0.705
Fasting glucose (mg/dL)	94.0±10.0	101.1±27.5	0.020*	87.7±7.3	89.4±16.3	0.235
2-hour glucose (mg/dL)	116.0±33.9	148.8±64.0	<0.001*	98.0±23.9	100.8±33.7	0.404
Haemoglobin A1c %	5.5±0.3	5.9±1.0	0.004*	5.4±0.3	5.4±0.4	0.646
HOMA-IR [a]	3.8±3.0	5.7±5.5	0.004*	1.9±2.1	1.9±1.1	0.866
Impaired glucose tolerance %	16%	32%	0.009*	6%	5%	0.603
Diabetes mellitus %	3%	16%	0.003*	1%	2%	0.378
Hormonal components						
LH (mIU/mL)	7.8±6.1	9.2±8.8	0.215	10.3±13.3	11.2±8.3	0.487
FSH (mIU/mL)	5.9±2.1	5.8±1.7	0.831	6.5±2.4	7.0±2.2	0.081
TSH (mIU/mL)	1.9±1.2	2.2±1.3	0.121	1.8±1.1	2.0±1.2	0.311
Prolactin (mIU/mL)	14.1±5.1	13.3±5.2	0.227	14.2±5.3	14.2±5.4	0.999
Liver function						
GOT [a] (IU/I)	24.0±10.1	32.0±20.9	0.001*	20.5±6.9	20.5±5.6	0.938
GPT [a] (IU/I)	24.0±13.9	41.5±34.5	<0.001*	16.7±9.7	17.9±9.3	0.291
Lipid profiles and blood pressure						
Cholesterol (mg/dL)	184.4±32.4	194.7±39.3	0.039*	179.0±33.1	186.0±32.3	0.069
Triglycerides (mg/dL)	103.6±60.5	142.6±125.3	0.006*	60.6±28.0	78.6±71.0	0.003*
HDL [a] (mg/dL)	44.8±12.5	42.6±11.3	0.156	60.0±14.8	59.8±15.1	0.914
LDL [a] (mg/dL)	118.7±26.5	125.7±32.0	0.087	100.0±28.6	105.2±28.4	0.118
Metabolism						
Metabolic Syndrome	43%	66%	<0.001*	3%	6%	0.337
Hypertension	47%	53%	0.354	11%	12%	0.695
HDL [a] < 50 mg/dl	74%	83%	0.091	25%	26%	0.914
Triglycerides ≥ 150 mg/dL	12%	32%	0.001*	1%	6%	0.012*
Waist > 80 cm	94%	96%	0.622	19%	19%	0.964
FPG [a] ≥ 100 mg/dL	19%	36%	0.007*	5%	7%	0.331

Note: Data are either mean ± SD or are percentage; * $p<0.05$,

[a]: Low Ferritin: serum ferritin level < 45.5 ng/mL, High Ferritin: serum ferritin level ≥ 45.5 ng/mL ; BMI: body mass index, PCOS: polycystic ovary syndrome, PCOM: polycystic ovary

morphology, SHBG: sex hormone-binding globulin, hsCRP: high-sensitivity C-reactive protein, Free androgen index (FAI) = T (nmol/l)X100/SHBG (nmol), DHEA-S: Dehydroepiandrosterone sulfate, 17-OH PRG :17-α-OH progesterone, HOMA-IR : The homeostasis model assessment insulin resistance index, GOT: Glutamic Oxaloacetic Transaminase, GPT: Glutamic Pyruvic Transaminase, HDL: high-density lipoprotein, LDL: low-density lipoprotein, FPG: Fasting plasma glucose

Obesity biomarkers in obese and non-obese women with polycystic ovary syndrome

Chin-I Chen MD[1], Ming-I Hsu MD[2] *, Shyh-Hsiang Lin PhD[3], Yuan-chin Ivan Chang PhD[4], Chun-Sen Hsu MD[2], Chii-Ruey Tzeng, MD[5]

[1]Department of Neurology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan,
[2]Department of Obstetrics and Gynaecology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan.
[3]School of Nutrition and Health Science, Taipei Medical University
[4]Institute of Statistical Science, Academia Sinica, Taipei, Taiwan
[5]Department of Obstetrics and Gynaecology, Taipei Medical University Hospital, Taipei Medical University, Taipei, Taiwan

Ming-I Hsu and Shyh-Hsiang Lin contributed equally to this work.

*Correspondence and reprint requests should be made to:
Ming-I Hsu, MD
Department of Obstetrics and Gynaecology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan
No.111, Sec. 3, Xinglong Rd., Taipei 11696, Taiwan.
Tel: +886-2-29307930 ext. 2501; Fax: +886-2-29300036
E-mail: hsumingi@yahoo.com.tw

Running title:
Obesity biomarkers in obese and non-obese women with PCOS

Abstract

STUDY QUESTION: Polycystic ovary syndrome (PCOS) and obesity are important conditions that are associated with insulin resistance in women. Several obesity biomarkers have been reported as indicators of insulin resistance and metabolic complications. The roles of various obesity biomarkers in obese/non-obese women with PCOS are still unclear.

SUMMARY ANSWER: Women with PCOS and obesity were characterised by a decreased adiponectin-to-leptin ratio. Non-obese women with PCOS had increased serum leptin levels, and obese women with PCOS were characterised by decreased serum adiponectin levels. Resistin levels, independent of body weight status, served as a predicative factor for insulin resistance in women with PCOS.

WHAT IS ALREADY KNOWN: Adiponectin, leptin, ghrelin, and resistin levels were associated with PCOS and obesity-linked complications. Women with PCOS had lower adiponectin levels and higher leptin levels than normal controls.

STUDY DESIGN, SIZE, DURATION: Medical records from our outpatient clinic were reviewed from Jan. 1, 2009, to Dec. 31, 2012. The candidates for the study cases were women who had been fully evaluated in terms of their levels of various androgens, cardiovascular risk, and metabolic components. Adiponectin, leptin, ghrelin, and resistin levels were also evaluated.
A total of 422 women were analysed in this study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Among the 422 studied patients were 224 women with PCOS and 198 women without PCOS. There were 182 women with obesity (BMI>25), which included 121 women with PCOS and 61 women without PCOS. The other 240 patients were non-obese women (BMI$\leqq$25), including 103 women with PCOS and 137 women without PCOS. Pearson correlation analysis was performed to assess insulin resistance, total testosterone levels, body mass index and various obesity biomarkers. The levels of obesity biomarkers in women with and without PCOS in the obese and non-obese groups were compared.

MAIN RESULTS AND THE ROLE OF CHANCE: Adiponectin/leptin ratios were significantly lower in women with PCOS than in normal control women. Significantly higher serum leptin levels were observed in non-obese women with PCOS and normal control subjects, but significantly lower serum adiponectin levels were observed in obese subjects. For women without PCOS, BMI was the only independent predictor

of insulin resistance. For women with PCOS, BMI and resistin were two independent predictors of insulin resistance. Resistin levels were negatively correlated with testosterone and LDL levels and positively correlated with fasting insulin levels, which might play controversial roles in medical complications in women of reproductive age. The role of ghrelin in insulin resistance in women with PCOS should not be determined by systemic plasma levels.

LIMITATIONS, REASONS FOR CAUTION: The major weakness of this study is that the women evaluated in the present study were recruited from the outpatient clinic of a tertiary care centre and do not reflect the true distribution of the general population. Therefore, these results should be applied to the general population with caution.

WIDER IMPLICATIONS OF THESE FINDINGS: Adipose tissue might play an important role in metabolic complications in women with PCOS. To study the impact of obesity biomarkers in women with PCOS; however, obese and lean women should be considered separately. Resistin levels might play controversial roles in medical complications of reproductive aged women.

STUDY FINDING/ COMPETING INTEREST(S):
This work was supported by the Taiwan National Science Council Grant NSC102-2629-B-038-001 and Taipei Medical University – Wan Fang Hospital Grant 100-TMU-WFH-02-4.

TRIAL REGISTRATION NUMBER:
This study was approved by the Institutional Review Board of Taipei Medical University – Wan Fang Hospital, Taipei, Taiwan with the identifier Hsu2013-TMU-JIRB201307021 and registered at ClinicalTrials.gov with the identifier NCT01989039.

Keywords: Adiponectin, leptin, resistin, ghrelin, obesity, PCOS

Introduction

Polycystic ovary syndrome (PCOS) is the most frequent endocrinopathy in reproductive-aged women, which is mainly characterised by oligoanovulation and hyperandrogenism. PCOS is associated with an adverse cardio-metabolic profile and consists of increased total or central adiposity and abnormal glucose metabolism (Lambrinoudaki *et al.,* 2011).

Obesity is characterised by adipocyte hypertrophy. Adipose tissue participates in the regulation of energy homeostasis as an important endocrine organ that secretes a number of biologically active adipokines (Kadowaki *et al.,* 2005). Dysregulated production or secretion of these adipokines because of adipose tissue dysfunction can contribute to obesity-linked complications (Scarpellini *et al.,* 2012). Adiponectin, leptin, resistin, and ghrelin are among the most investigated factors that may impact obese women. Adiponectin is a recently identified adipocyte-derived collagen-like protein. It is exclusively expressed in adipose tissue and is released into the circulation (Stefan *et al.,* 2002). Adiponectin levels hold great promise for use in clinical applications as a potent indicator of underlying metabolic complications (Trujillo *et al.,* 2005). Leptin, an adipocyte-derived hormone encoded by 'ob' gene, relays metabolic signals to the neuronal networks in the brain to modulate the hypothalamo-pituitary-ovarian axis (Chakrabarti J,2013). The leptin- neuropeptide Y axis was suggested for its implications in reproductive disturbance (Jacobs *et al.,* 1999). Resistin was originally described as an adipocyte-specific hormone. Some studies indicate that resistin plays important regulatory roles apart from its role in insulin resistance and diabetes (Jamaluddin *et al.,* 2012). Ghrelin is a natural ligand of the growth hormone secretagogue receptor and is a potent stimulant of GH secretion. Ghrelin plays a role in regulating glucose metabolism and energy balance. Polymorphisms in preproghrelin and the ghrelin gene could be responsible for obesity and insulin resistance, and low ghrelin levels are observed in some individuals (Wang *et al.,* 2009). All of these obesity biomarkers might be used to indicate metabolic complications in obese women.

Several studies have suggested that the above-mentioned biomarkers contributed to insulin resistance in women with PCOS. Wang suggested that low serum adiponectin and high serum resistin levels might play important roles in the pathogenesis of insulin resistance in PCOS patients (Wang *et al.,* 2010). Pehlivanov reported a positive correlation between serum leptin levels and the clinical and hormonal indices of insulin resistance. Pehlivanov suggested that hyperleptinaemia is due to leptin

resistance and may be a characteristic of PCOS (Pehlivanov *et al.,* 2009). Isolated adipocytes from women with PCOS express higher mRNA concentrations of some adipokines involved in cardiovascular risk and insulin resistance (Garruti *et al.,* 2009). Adipose tissue obtained from women with PCOS revealed that resistin gene overexpression in adipocytes may be a local determining factor in PCOS pathogenesis (Seow *et al.,* 2004). Skommer proposed that the presence of ghrelin in PCOS and normal ovaries may have an autocrine/paracrine modulatory effect on ovary functions and a local significance in PCOS aetiology (Skommer *et al.,* 2005). All of above studies indicated an important role for adipose biomarkers related to insulin resistance in women with PCOS.

Obesity is actually associated with increased adipose and plasma leptin level and lower adiponectin expression (Svendsen *et al.,* 2012). Obesity might affect the pathogenesis of adipose tissue hormones. Studies on an adipose-related factor in women with PCOS have been controversial. Olszanecka-Glinianowicz suggested that obese but not normal-weight women with PCOS have lower adiponectin levels, whereas resistin concentrations did not differ in normal-weight and obese PCOS subjects compared with control subjects (Olszanecka-Glinianowicz *et al.,* 2011).

Because adipose tissue is a key endocrine organ (Galic *et al.,* 2010), any correlation between adipokines and insulin resistance in women with PCOS should be considered in lean and obese women separately. Thus, we evaluated the above obesity biomarkers in obese and non-obese women with and without PCOS.

Materials and Methods

This study was approved by the Institutional Review Board of Taipei Medical University – Wan Fang Hospital, Taipei, Taiwan with the identifier Hsu2013-TMU-JIRB201307021 and registered at ClinicalTrials.gov with the identifier NCT01989039. We retrospectively reviewed the medical records of female patients who visited our Reproductive Endocrinology Clinic from Jan. 1, 2009, to Dec. 31, 2012.

The study population

Study data

Women were initially included who had a complete set of anthropometric measurements as well as clinical and biochemical data regarding insulin resistance parameters and obesity biomarkers. The subjects' medical histories included a detailed menstrual and medical/surgical history, anthropometric measurements (weight, height,

waist, and hip), and blood pressure. The dates and assays performed for blood sampling have been previously described (Liang *et al.*, 2011). The following data were collected and calculated: (1) obesity hormone levels: adiponectin, leptin, ghrelin, and resistin; (2) serum androgen levels including total testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEA-S), and the free androgen index (FAI), which was calculated as follows: FAI = total testosterone (nmol/l)× 100/ sex hormone binding globulin (SHBG) (nmol/l); (3) insulin sensitivity and glucose tolerance assessments including fasting insulin and glucose levels, two-hour glucose levels and the homeostasis model assessment insulin resistance index (HOMA); (4) lipid profiles including total cholesterol, triglyceride, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels. Furthermore, metabolic syndrome risk, impaired glucose tolerance and diabetes were evaluated in every subject. The waist-to-hip ratio (WHR) was defined as the waist circumference/hip circumference. Body mass index (BMI) was defined as the body weight in kilograms divided by the body height in meters squared (kg/m^2). Obesity was defined as BMI $\geq$ 25 kg/m^2 (Asia-Pacific Perspective, 2002). An ovarian pelvic ultrasonograph, preferably transvaginal, was performed to detect polycystic ovaries.

The following subjects were excluded from the study populations: (1) women who had been diagnosed with malignant tumours, Asherman's syndrome, Mullerian agenesis, or chromosomal anomalies; (2) females younger than 13 or older than 49 years old; and (3) women who received hormones/drugs for major medical diseases within the previous three months.

Serum FSH and LH levels were measured using enzyme immunoassays (AxSym System, Abbott Laboratories, IL, USA); serum total testosterone, DHEA-S, and androstenedione concentrations were measured by radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX), SHBG levels were measured using an electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics, Indianapolis, IN, USA); fasting insulin concentrations were measured by radioimmunoassay (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA), and fasting glucose levels were measured using the Beckman Coulter Synchron LX (Fullerton, CA, USA). Adiponectin, leptin and ghrelin were measured by RIA (LINCO Research, Inc. St. Charles, Missouri, MO, USA), and resistin was measured by enzyme immunoassay (R&D Systems, Inc. Minneapolis, MN, USA).

PCOS was diagnosed according to the Androgen Excess and PCOS Society criteria (Azziz *et al.*, 2009), which requires hyperandrogenism (hirsutism and/or biochemical) and ovarian dysfunction (oligo-anovulation and /or polycystic ovaries).

The definitions of oligo-anovulation and polycystic ovaries have been described in detail previously (Liang *et al.*, 2011). Hyperandrogenism (HA) was defined as hirsutism and/or biochemical hyperandrogenaemia (BioHA). Biochemical hyperandrogenaemia was defined as a total serum testosterone value ≥ 2.78 nmol/L (0.8 ng/mL, normal range for female adults is 0.1-0.8 ng/mL), and/or FAI ≥6.53. Hirsutism was defined as a modified Ferriman–Gallwey (mF-G) score of ≥6.

The insulin sensitivity index was evaluated by the homeostasis model assessment insulin resistance index (HOMA) using the following formula:

HOMA = [fasting insulin (μU/mL) × fasting glucose (mg/dL)]/405

World Health Organization 2006 diagnostic criteria for diabetes were used (fasting plasma glucose [FPG] ≥ 7 nmol/L (126 mg/dL) or 2-hour plasma glucose ≥ 11.1 nmol/L (200 mg/dL)). Impaired glucose tolerance (IGT) was defined as 2-hour glucose levels of 7.77 – 11.1 nmol/L (140–199 mg/dL) in the 75-g oral glucose tolerance test. In women with IGT, FPG levels should be less than 7 nmol/L.

Metabolic syndrome (MBS) was defined (2005 National Cholesterol Education Program - Adult Treatment Panel III) as the presence of at least three of the following criteria: abdominal obesity (waist circumference > 80 cm in women), serum triglyceride levels ≥ 1.7 nmol/L (150 mg/dL), serum HDL levels < 1.3 nmol/L (50 mg/dL), systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg, and fasting plasma glucose levels ≥5.6 nmol/L (100 mg/dL).

A total of 543 women were primarily included for evaluation in the study. Of these, 121 cases were excluded due to premature ovarian failure (N=23) and hyperprolactinaemia (N=98). Finally, 422 cases were analysed in this study (Figure 1).

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL). We evaluated the correlation between serum HOMA IR, total testosterone and inflammatory markers using Pearson's correlation coefficients with the two-tailed method (Table 1). The data are represented as the mean ± standard deviation in Table 2. We used chi-square and Fisher's exact test to compare categorical variables and ANOVA to compare continuous variables in Tables 2 and 3. Multiple regression models were used to examine the relationships between HOMA and the associated risk factors. Differences between groups were considered

1 significant if the corresponding p-value was less than 0.05.
2

Results:

Among the 422 studied patients, 224 women had PCOS and 198 women did not have PCOS. There were 182 obese women (BMI>25), of which 121 had PCOS, and 61 did not. The others were 240 non-obese women (BMI≦25), of which 103 women had PCOS, and 137 did not.

Table 1 demonstrated the correlation between insulin resistance (HOMA), total testosterone levels, and body mass index (BMI) with various obesity biomarkers. The results demonstrated that adiponectin was negatively correlated with HOMA, BMI, total testosterone, triglyceride, LDL and leptin levels (all $p<0.001$); conversely, leptin levels were significantly positively correlated with HOMA, BMI, total testosterone, triglyceride, and LDL levels (all $p<0.001$). Furthermore, ghrelin levels were negatively correlated with BMI ($p<0.001$) and total testosterone levels ($p=0.012$). Resistin levels were negatively correlated with total testosterone ($p=0.012$) and LDL levels ($p=0.033$) and were positively correlated with fasting insulin levels ($p=0.050$). Adiponectin, leptin and ghrelin levels were highly correlated with BMI; however, resistin levels were not correlated with BMI.

After adjusting for BMI, adiponectin levels were negatively correlated with triglyceride levels (μ =-0.176, $p<0.001$) and positively with resistin (μ =0.101, $p=0.039$) and HDL (μ =0.358, $p<0.001$) levels. Leptin levels were positively correlated with total testosterone levels (μ =0.207, $p<0.001$). Resistin levels were positively correlated with HOMA (μ =0.135, $p=0.006$) and fasting insulin levels (μ =0.143, $p=0.003$) and were negatively correlated with total testosterone (μ =-0.103, $p=0.035$) and LDL levels (μ =-0.097, $p=0.048$).

To consider the impact of obesity on women with and without PCOS separately, table 2 provides the obesity biomarkers in obese and non-obese women among women with or without PCOS. The results demonstrate that adiponectin and leptin levels as well as the adiponectin/leptin ratio was significantly different in obese compared with non-obese women in the PCOS and non-PCOS groups. However, serum resistin levels were not different in obese and non-obese women.

Table 3 presents a comparison between obese and non-obese PCOS and non-PCOS women separately. The adiponectin to leptin ratios were significantly lower in women with than without PCOS. To compare with women with non-PCOS, Obese women with PCOS had lower serum adiponectin levels than women without PCOS, but non-obese women did not. Conversely, non-obese women with PCOS had higher

serum leptin levels than those without PCOS, but obese women did not. However, ghrelin and resistin levels were not significantly different in obese or lean women with or without PCOS. Insulin resistance prevalence was similar between women that had PCOS and those that did not.

A multiple regression model was used to evaluate obesity biomarkers in order to predict insulin resistance (homeostasis model assessment) under various conditions. HOMA was used as the dependent variable, and BMI, total testosterone, adiponectin, leptin, ghrelin, and resistin levels were used as independent variables. For non-PCOS women, BMI was the only independent predictor of insulin resistance, which explained approximately 47% of HOMA variability. For women with PCOS, BMI and resistin levels were two independent insulin resistance predictors that explained approximately 57 % of HOMA variability.

Discussion:

The state of the cardiovascular system in women with PCOS might contribute to different factors including insulin resistance, androgen status and BMI (Samy *et al.,* 2009). Excess androgen should be the most important component of PCOS (Azziz *et al.,* 2006). Serum total testosterone levels could be considered to be indicative of hyperandrogenism severity. HOMA was also considered to be an indicator of insulin resistance, and BMI was an indicator of obesity. The first part of this study was to evaluate the correlation between obesity biomarkers and HOMA, BMI and total testosterone levels. Adiponectin, leptin, and ghrelin levels were highly BMI-dependent, but resistin levels were not. Adiponectin and leptin-mediated reversal of insulin resistance and lipid profiles was very clear. However, the models of resistin action were complicated. Resistin levels were negatively correlated with total testosterone and LDL levels, similar to adiponectin. Resistin levels were positively correlated with fasting insulin levels, similar to leptin levels.

Adipocytes are considered endocrine cells that synthesize and release molecules (adipokines) that play an endocrine/paracrine role. Our results demonstrate that adiponectin levels were negatively associated with BMI, insulin resistance, and total testosterone. Conversely, leptin reversed the above-mentioned reaction and was negatively correlated with adiponectin levels. Svendsen suggested that PCOS did not have an independent effect on adipose tissue expression of leptin, adiponectin, IL-6 or circulating adipocytokine levels (Svendsen *et al.,* 2012). Our results demonstrated that obese women with or without PCOS had lower adiponectin and higher leptin levels than lean women. Our study also confirmed a previous report that the adiponectin/leptin ratio was a marker of women with PCOS (Golbahar *et al.,* 2012). Furthermore, our results were the first to suggest opposing roles of adiponectin and leptin in obese and lean women with PCOS. Compared with normal controls, lean but not obese women with PCOS presented with significantly higher serum leptin levels. Conversely, obese but not lean women with PCOS presented with lower serum adiponectin levels than normal controls. Our results also demonstrated a negative correlation between total testosterone and adiponectin levels; therefore, obese women with PCOS demonstrated significantly lower adiponectin levels than obese controls. Our study also confirmed that adiponectin levels and insulin resistance are significantly correlated, and low adiponectin levels may be involved in insulin resistance in PCOS patients, as was previously reported (Wang *et al.,* 2011). Fasting insulin levels in obese women with PCOS were significantly higher than those of the obese controls. Obesity-linked adiponectin down-regulation might be a mechanism whereby obesity can cause insulin resistance and diabetes (Kadowaki *et al.,* 2005);

furthermore, PCOS might enhance this condition. This study also indicates that circulating adiponectin levels could be treated as an insulin resistance biomarker (Trujillo *et al.,* 2005) and that adipocytokine and metabolic biomarker levels are significantly correlated (Wang *et al.,* 2011). Furthermore, our study demonstrated a strong positive correlation between adiponectin and HDL levels even after adjusting for BMI, which might imply that adiponectin has a favourable effect on the lipid profile.

The role of resistin in PCOS and obesity are controversial. Some studies have suggested that resistin plays important regulatory roles in a variety of biological processes apart from its role in insulin resistance and diabetes (Chu *et al.,* 2009; Jamaluddin *et al.,* 2012), and higher resistin levels have been reported in women with PCOS compared with controls (Yilmaz *et al.,* 2009). However, based on Pearson's analysis, our results did not demonstrate a correlation between resistin levels and BMI or HOMA. Furthermore, using original data analysis, serum resistin concentrations were not different in obese or lean women with or without PCOS. Interestingly, regression analysis demonstrated that BMI and resistin were the only two independent predictors for insulin resistance in women with PCOS. Further analysis determined that although resistin levels were not correlated with insulin resistance in the original data, the positive correlation between resistin levels and HOMA and between resistin levels and fasting insulin levels became highly significant after adjusting for BMI.

Although resistin is known to be an adipose tissue-specific secreted factor, resistin gene expression was barely detectable in mature adipocytes and is highly expressed in preadipocytes. Preadipocyte differentiation to adipocytes was associated with time-dependent down-regulation of resistin gene expression (Janke *et al.,* 2002). Although resistin expression was initially defined in adipocytes, the major cell populations that express and produce in humans are mononuclear leukocytes, macrophages, splenic and bone marrow cells (Patel *et al.,* 2003; Fain *et al.,* 2003). Most previous studies confirmed that there was no correlation between BMI and serum resistin levels (Güven *et al.,* 2010, Olszanecka-Glinianowicz *et al.,* 2011; Arikan *et al.,* 2010; Seow *et al.,* 2004; Zhang *et al.,* 2011). Furthermore, the association between serum resistin levels and insulin resistance could not be proven by most clinical studies (Güven *et al.,* 2010, Olszanecka-Glinianowicz *et al.,* 2011; Arikan *et al.,* 2010; Zhang *et al.,* 2011). Olszanecka-Glinianowicz studied 41 women with PCOS and 16 control women (Olszanecka-Glinianowicz *et al.,* 2011), Arikan studied 31 non-obese women with PCOS and 25 controls (Arikan *et al.,* 2010), Pangaribuan studied 14 obese women with PCOS, 10 non-obese women with PCOS

and 18 non-obese controls (Pangaribuan *et al.*, 2011), Güven studied 22 adolescents with PCOS and 16 controls (Güven *et al.*, 2010). None of the above studies could detect a difference in serum resistin levels between women with PCOS and controls. Our study included 422 women and contained 224 women with PCOS and 198 women without PCOS, and we also did not discover any difference in serum resistin levels between women with PCOS and controls in our original data. Furthermore, the association between resistin levels and insulin resistance could only be found after BMI adjustment in our study.

Serum resistin levels and metabolic disturbances were complicated. The serum resistin level results positively correlated with fasting insulin levels and negatively correlated with LDL levels, which demonstrated controversial conditions in our knowledge of medical complications. Contrary to some previous reports, our results demonstrated that serum resistin levels positively correlated with adiponectin and negatively correlated with total testosterone levels. The correlation between adiponectin and resistin levels are still controversial. Lewandowski studied 19 patients and suggested that serum adiponectin and resistin concentrations in women with PCOS were negatively correlated (Lewandowski *et al.*, 2005). Güven studied 22 adolescents with PCOS and 16 controls and determined no differences between serum adiponectin and resistin concentrations in girls with PCOS (Güven *et al.*, 2010). A negative correlation between resistin and free testosterone levels was reported in a previous study (Bideci *et al.*, 2008). The positive correlation of resistin levels with adiponectin levels and the negative correlation of resistin levels with total testosterone levels might provide the different aspects for resistin insulin resistance pathogenesis in women with PCOS. The association between resistin levels and insulin resistance became significant after BMI adjustment. Resistin should be an important predictor of insulin resistance, which is independent of BMI. The role of resistin in women with PCOS is worth exploring further.

Ghrelin might be related to metabolic and androgenic changes in patients with PCOS (Sağsöz *et al.*, 2009). A major focus of research with ghrelin has been primarily related to food intake regulation and its accompanying endocrine function (Karmiris *et al.*, 2008). However, reports on ghrelin in women with PCOS are still controversial; Krentz reported that ghrelin levels were reduced in women with PCOS compared with unaffected control women (Krentz *et al.*, 2009). Waśko reported that women with PCOS had higher ghrelin levels compared with controls (Waśko *et al.*, 2004). Orio demonstrated that plasma ghrelin concentrations are not different in women with PCOS compared with weight-matched controls and were inversely correlated with

BMI (Orio *et al.*, 2003). Ozgen suggested that the low ghrelin level in obese PCOS patients is evidence of the role of ghrelin in the pathogenesis of this syndrome (Ozgen *et al.*, 2010). Our results demonstrated that serum ghrelin levels were negatively correlated with BMI and leptin levels, and obese women with PCOS had lower ghrelin levels than lean women with PCOS. Furthermore, our study could not determine any differences in serum ghrelin levels among obese and lean women with PCOS and controls. Obesity is a more predominant factor for serum ghrelin level determination than PCOS; however, the decrease in ghrelin levels seems more significant in obese than lean women with PCOS.

Finally, we can conclude the following:

Women with PCOS presented with a decreased adiponectin-to-leptin ratio. Compared with normal controls, women with PCOS display increased serum leptin levels, but this was observed only in lean and not obese subjects. Conversely, decreased serum adiponectin levels were observed in obese but not lean subjects.

For women without PCOS, BMI was the only independent predictor of insulin resistance. For women with PCOS, BMI and resistin levels were two independent predictors of insulin resistance.

Resistin, independent of BMI, negatively correlated with testosterone and LDL levels and positively correlated with fasting insulin levels, which might play a controversial role in the medical complications of reproductive aged women.

The correlation between resistin levels and insulin resistance were not significant; however, the positive correlation between resistin levels and insulin resistance became significant after BMI adjustment.

The roles of ghrelin in insulin resistance pathogenesis for women with PCOS should not be determined systemically via plasma levels.

Reference:

1. Lambrinoudaki I. Cardiovascular risk in postmenopausal women with the polycystic ovary syndrome. *Maturitas* 2011; **68**:13-6.
2. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev.* 2005;**26**:439-51.
3. Scarpellini E, Tack J. Obesity and metabolic syndrome: an inflammatory condition. *Dig Dis.* 2012;**30**:148-53.
4. Stefan N, Stumvoll M. Adiponectin--its role in metabolism and beyond. *Horm Metab Res.* 2002;**34**:469-74.
5. Trujillo ME, Scherer PE. Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. *J Intern Med.* 2005;**257**:167-75.

6. Chakrabarti J Serum leptin level in women with polycystic ovary syndrome: correlation with adiposity, insulin, and circulating testosterone. *Ann Med Health Sci Res.* 2013;**3**:191-6.
7. Jacobs HS, Conway GS. Leptin, polycystic ovaries and polycystic ovary syndrome. *Hum Reprod Update.* 1999;**5**:166-71.
8. Jamaluddin MS, Weakley SM, Yao Q, Chen C. Resistin: functional roles and therapeutic considerations for cardiovascular disease. *Br J Pharmacol.* 2012;**165**:622-32.
9. Wang K, Wang L, Zhao Y, Shi Y, Wang L, Chen ZJ. No association of the Arg51Gln and Leu72Met polymorphisms of the ghrelin gene and polycystic ovary syndrome. *Hum Reprod.* 2009;**24**:485-90.
10. Wang Y, Xie X, Zhu W Serum adiponectin and resistin levels in patients with polycystic ovarian syndrome and their clinical implications. *J Huazhong Univ Sci Technolog Med Sci.* 2010;**30**:638-42.
11. Pehlivanov B, Mitkov M. Serum leptin levels correlate with clinical and biochemical indices of insulin resistance in women with polycystic ovary syndrome. *Eur J Contracept Reprod Health Care.* 2009;**14**:153-9.
12. Garruti G, Depalo R, Vita MG, Lorusso F, Giampetruzzi F, Damato AB, Giorgino F. Adipose tissue, metabolic syndrome and polycystic ovary syndrome: from pathophysiology to treatment. *Reprod Biomed Online.* 2009;**19**:552-63.
13. Seow KM, Juan CC, Wu LY, Hsu YP, Yang WM, Tsai YL, Hwang JL, Ho LT. Serum and adipocyte resistin in polycystic ovary syndrome with insulin resistance. *Hum Reprod.* 2004;**19**:48-53.
14. Skommer J, Katulski K, Poreba E, Meczekalski B, Slopień R, Plewa R, Gozdzicka-Józefiak A, Warenik-Szymankiewicz A. Gherlin expression in women with polycystic ovary syndrome--a preliminary study. *Eur J Gynaecol Oncol.* 2005;**26**:553-6.
15. Svendsen PF, Christiansen M, Hedley PL, Nilas L, Pedersen SB, Madsbad S. Adipose expression of adipocytokines in women with polycystic ovary syndrome. *Fertil Steril.* 2012;**98**:235-41.
16. Olszanecka-Glinianowicz M, Kuglin D, Dąbkowska-Huć A, Skałba P. Serum adiponectin and resistin in relation to insulin resistance and markers of hyperandrogenism in lean and obese women with polycystic ovary syndrome. *Eur J Obstet Gynecol Reprod Biol.* 2011;**154**:51-6.
17. Galic S, Oakhill JS, Steinberg GR. Adipose tissue as an endocrine organ. *Mol Cell Endocrinol.* 2010 25;**316**:129-39.
18. Liang SJ, Hsu CS, Tzeng CR, Chen CH, Hsu MI. Clinical and biochemical presentation of polycystic ovary syndrome in women between the ages of 20 and

40. *Hum Reprod.* 2011;**26**:3443-9.
19. The Asia-Pacific Perspective. Redefining obesity and treatment, WHO Western Pacific Region. International Association for the study of obesity. 2002
20. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al; Task Force on the Phenotype of the Polycystic Ovary Syndrome of The Androgen Excess and PCOS Society. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. *Fertil Steril.* 2009;**91**:456-88.
21. Samy N, Hashim M, Sayed M, Said M. Clinical significance of inflammatory markers in polycystic ovary syndrome: their relationship to insulin resistance and body mass index. *Dis Markers.* 2009;**26**:163-70.
22. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ, Taylor AE, Witchel SF; Androgen Excess Society. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. *J Clin Endocrinol Metab.* 2006;**91**:4237-45.
23. Golbahar J, Das NM, Al-Ayadhi MA, Gumaa K. Leptin-to-adiponectin, adiponectin-to-leptin ratios, and insulin are specific and sensitive markers associated with polycystic ovary syndrome: a case-control study from Bahrain. *Metab Syndr Relat Disord.* 2012;**10**:98-102.
24. Wang Q, Guo T, Tao Y, Wang Q, Song Y, Huang W. Association between serum adipocyte factor level and insulin resistance in polycystic ovarian syndrome. *Gynecol Endocrinol.* 2011;**27**:931-4.
25. Chu Y, Cui Q, Feng G, Song Z, Jiang X. The expression of resistin in adipose tissues of patients with polycystic ovary syndrome and insulin resistance. J *Huazhong Univ Sci Technolog Med Sci.* 2009;**29**:642-5.
26. Yilmaz M, Bukan N, Demirci H, Oztürk C, Kan E, Ayvaz G, Arslan M. Serum resistin and adiponectin levels in women with polycystic ovary syndrome. *Gynecol Endocrinol*. 2009;**25**:246-52.
27. Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM. Resistin gene expression in human adipocytes is not related to insulin resistance. *Obes Res.* 2002;**10**:1-5.
28. Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, Macphee CH, Smith SA. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem Biophys Res Commun.* 2003 10;**300**:472-6.
29. Fain JN, Cheema PS, Bahouth SW, Lloyd Hiler M. Resistin release by human adipose tissue explants in primary culture. *Biochem Biophys Res Commun.* 2003 17;**300**:674-8.

30. Güven A, Ozgen T, Aliyazicioğlu Y. Adiponectin and resistin concentrations after glucose load in adolescents with polycystic ovary syndrome. *Gynecol Endocrinol.* 2010;**26**:30-8.
31. Arikan S, Bahceci M, Tuzcu A, Kale E, Gökalp D. Serum resistin and adiponectin levels in young non-obese women with polycystic ovary syndrome. *Gynecol Endocrinol.* 2010;**26**:161-6.
32. Zhang J, Zhou L, Tang L, Xu L. The plasma level and gene expression of resistin in polycystic ovary syndrome. *Gynecol Endocrinol.* 2011;**27**:982-7.
33. Pangaribuan B, Yusuf I, Mansyur M, Wijaya A. Serum adiponectin and resistin in relation to insulin resistance and markers of hyperandrogenism in lean and obese women with polycystic ovary syndrome. *Ther Adv Endocrinol Metab.* 2011;**2**:235-45.
34. Lewandowski KC, Szosland K, O'Callaghan C, Tan BK, Randeva HS, Lewinski A. Adiponectin and resistin serum levels in women with polycystic ovary syndrome during oral glucose tolerance test: a significant reciprocal correlation between adiponectin and resistin independent of insulin resistance indices. *Mol Genet Metab.* 2005;**85**:61-9.
35. Bideci A, Camurdan MO, Yeşilkaya E, Demirel F, Cinaz P. Serum ghrelin, leptin and resistin levels in adolescent girls with polycystic ovary syndrome. J *Obstet Gynaecol Res.* 2008;**34**:578-84.
36. Sağsöz N, Orbak Z, Noyan V, Yücel A, Uçar B, Yildiz L. The effects of oral contraceptives including low-dose estrogen and drospirenone on the concentration of leptin and ghrelin in polycystic ovary syndrome. *Fertil Steril.* 2009;**92**:660-6.
37. Karmiris K, Koutroubakis IE, Kouroumalis EA. Leptin, adiponectin, resistin, and ghrelin--implications for inflammatory bowel disease. *Mol Nutr Food Res.* 2008;**52**:855-66.
38. Krentz AJ, Barrett-Connor E. Reduced serum ghrelin in a putative postmenopausal polycystic ovary syndrome phenotype. *Fertil Steril.* 2009;**92**:1753-4.
39. Waśko R, Komarowska H, Warenik-Szymankiewicz A, Sowiński J. Elevated ghrelin plasma levels in patients with polycystic ovary syndrome. *Horm Metab Res.* 2004;**36**:170-3.
40. Orio F Jr, Lucidi P, Palomba S, Tauchmanova L, Cascella T, Russo T, Zullo F, Colao A, Lombardi G, De Feo P. Circulating ghrelin concentrations in the polycystic ovary syndrome. *J Clin Endocrinol Metab.* 2003;**88**:942-5.

41. Ozgen IT, Aydin M, Guven A, Aliyazicıoglu Y. Characteristics of polycystic ovarian syndrome and relationship with ghrelin in adolescents. *J Pediatr Adolesc Gynecol.* 2010;**23**:285-9.

Legends

Figure 1: Flow chart of patient selection for this study

Table 1 Pearson correlation between insulin resistance, total testosterone, and body mass index with various obesity biomarkers

Table 2 A comparison of obesity biomarkers among obese with non-obese women with or without PCOS

Table 3 A comparison of various biomarkers in obese and non-obese women with and without PCOS

REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

RESEARCH Open Access

1,25-Dihydroxyvitamin D3 increases testosterone-induced 17beta-estradiol secretion and reverses testosterone-reduced connexin 43 in rat granulosa cells

Ching-Tien Lee[1], Jiz-Yuh Wang[2], Kuang-Yi Chou[3] and Ming-I Hsu[4*]

Abstract

Background: Aromatase converts testosterone into 17beta-estradiol in granulosa cells, and the converted 17beta-estradiol contributes to follicular maturation. Additionally, excessive testosterone inhibits aromatase activity, which can lead to concerns regarding polycystic ovary syndrome (PCOS). Generally, 1,25-dihydroxyvitamin D3 (1,25D3) supplements help to improve the symptoms of PCOS patients who exhibit low blood levels of 1,25D3. Therefore, this study investigated the interaction effects of 1,25D3 and testosterone on estrogenesis and intercellular connections in rat granulosa cells.

Methods: Primary cultures of granulosa cells were treated with testosterone or testosterone plus 1,25D3, or pre-treated with a calcium channel blocker or calcium chelator. Cell lysates were subjected to western blot analysis to determine protein and phosphorylation levels, and 17beta-estradiol secretion was examined using a radioimmunoassay technique. Cell viability was evaluated by MTT reduction assay. Connexin 43 (Cx43) mRNA and protein expression levels were assessed by qRT-PCR, western blot, and immunocytochemistry.

Results: Testosterone treatment (0.1 and 1 microg/mL) increased aromatase expression and 17beta-estradiol secretion, and the addition of 1,25D3 attenuated testosterone (1 microg/mL)-induced aromatase expression but improved testosterone-induced 17beta-estradiol secretion. Furthermore, testosterone-induced aromatase phosphotyrosine levels increased at 10 min, 30 min and 1 h, whereas 1,25D3 increased the longevity of the testosterone effect to 6 h and 24 h. Within 18–24 h of treatment, 1,25D3 markedly enhanced testosterone-induced 17beta-estradiol secretion. Additionally, pre-treatment with a calcium channel blocker nifedipine or an intracellular calcium chelator BAPTA-AM reduced 1,25D3 and testosterone-induced 17beta-estradiol secretion. Groups that underwent testosterone treatment exhibited significantly increased estradiol receptor beta expression levels, which were not affected by 1,25D3. Neither testosterone nor 1,25D3 altered 1,25D3 receptor expression. Finally, at high doses of testosterone, Cx43 protein expression was decreased in granulosa cells, and this effect was reversed by co-treatment with 1,25D3.

Conclusions: These data suggest that 1,25D3 potentially increases testosterone-induced 17beta-estradiol secretion by regulating aromatase phosphotyrosine levels, and calcium increase is involved in both 1,25D3 and testosterone-induced 17beta-estradiol secretion. 1,25D3 reverses the inhibitory effect of testosterone on Cx43 expression in granulosa cells.

Keywords: 17beta-estradiol, Estrogenesis, Connexin 43, Testosterone, 1,25-dihydroxyvitamin D3, Polycystic ovary syndrome, Calcium, Granulosa cells

* Correspondence: hsumingi@yahoo.com.tw
[4]Department of Obstetrics and Gynecology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan
Full list of author information is available at the end of the article

Background

Biosynthesis of 17β-estradiol from androgen precursors is catalysed by the enzyme aromatase, which is expressed from the *cyp19* gene and exists in the ovaries, placenta, testes, breasts, brain, fat, liver and muscles [1]. 17β-Estradiol production and follicular development are controlled by the expression level and activity of aromatase [2]. During follicular growth, aromatase mRNA expression levels in granulosa cells from dominant follicles and 17β-estradiol levels in the follicular fluid are significantly increased [3,4].

Various studies have demonstrated the role of androgens in stimulating follicular development. Androgen receptors (AR) are observed in primary follicles and advanced-stage follicles, and are detected in the granulosa cells of primordial follicles [5]. Androgens have been shown to stimulate the growth of small antral follicles and inhibit apoptosis of preovulatory follicles in primate ovaries [6,7], whereas AR-knockout mice exhibit greatly increased apoptosis of granulosa cells in preovulatory follicles [8]. Testosterone and dihydrotestosterone (DHT) stimulate the growth of cultured follicles, increase the number of follicles and increase granulosa cell proliferation in mammalian cells [6,7]. Testosterone and androstenedione significantly increase the abundance of aromatase mRNA and the accumulation of 17β-estradiol [2,9,10]. However, excessive testosterone is involved in polycystic ovary syndrome (PCOS), which is the most common endocrine disorder in females and is associated with arrested follicular development and the failure to select a dominant follicle [11]. Deficiency of 1,25-dihydroxyvitamin D_3 ($1,25D_3$), an active form of vitamin D, is a common risk factor in patients with PCOS [12,13]. A daily $1,25D_3$ supplement enhances the intestinal absorption of calcium and alleviates both PCOS symptoms and gonadal dysfunction in $1,25D_3$ receptor (VDR)-null mutant mice [14].

$1,25D_3$ plays important roles in calcium homeostasis, bone metabolism, and cell differentiation, proliferation, and apoptosis. Immunohistochemistry assays have demonstrated that VDR localizes to the follicles and predominantly exists in the nuclei of granulosa cells, suggesting that it has some role in reproduction [15]. Recent studies have demonstrated that aromatase activity and expression level are low in the ovaries of VDR-null mutant mice, but the activity is increased to 60% of wild-type levels by calcium supplementation in the normal range. Circulating 17β-estradiol levels are also low in VDR-null mutant mice and do not rise after calcium supplementation [16]. Taken together, these results indicate that $1,25D_3$ plays a role in estrogenesis that is only partially mediated by extracellular calcium homeostasis.

It has recently been shown that signalling via $1,25D_3$/VDR-mediated protein tyrosine phosphorylation occurs in bone, intestine, muscle and cancer cells [17]. The fast nongenomic responses of $1,25D_3$/VDR in muscle cells involve the phosphotyrosine form of a downstream protein, c-Src [18,19]. Interestingly, c-Src is also downstream of the estradiol/membrane-associated estradiol receptor (ER) in the stimulation of aromatase activity. The phosphorylation of aromatase at tyrosine 361 is crucial in the up-regulation of aromatase activity through the estradiol/membrane-associated ER and c-Src signalling in mammalian cell lines [20]. The AR is also localized in the plasma membrane and is associated with Src after testosterone treatment in Sertoli cells [21].

A gap junction is a type of intercellular connection that enables the transfer of various small molecules and ions between cells. Such transfers between granulosa cells and oocytes have been implicated in playing important roles in follicular development and oocyte growth [22]. Gap junctions between granulosa cells contain abundant levels of connexin (Cx) 43, which is present at every stage of follicular growth [23]. The level of Cx43 protein expression increases during follicular development and decreases with follicular atresia [24]. Oocytes from Cx43-null mice failed to reach meiotic maturation, resulting in infertility [24]. Wu et al. demonstrated that excess DHT reduces Cx43 expression and impairs communication between granulosa cells [25]. Additionally, $1,25D_3$ increases Cx43 protein levels and Cx43 mRNA stability via the nuclear VDR in human skin fibroblasts [26].

However, no studies have investigated the effect of the testosterone and $1,25D_3$ interaction on aromatase expression and phosphorylation in granulosa cells. Additionally, the effects of $1,25D_3$ and testosterone-regulated 17β-estradiol production and intercellular communication in granulosa cells have not been fully elucidated. We therefore investigated the interactions of $1,25D_3$ and testosterone to clarify their effects on aromatase protein expression and tyrosine phosphorylation, 17β-estradiol secretion and Cx43 protein expression in cultured ovarian granulosa cells.

Methods

Animal preparation

Immature female Sprague–Dawley rats were housed in plastic cages and maintained on a 12 h light/12 h dark cycle (light on at 6:00 a.m.) with food and water available continuously. The experimental procedures were consistent with the Guidelines of Animal Use and Care from the National Institute of Health and were approved by the Institutional Animal Care and Use Committee of Taipei Medical University-Wang Fang Hospital, Taiwan. Immature female rats at 23–24 days of age were injected with 15 IU PMSG (Sigma, St. Louis, MO, USA) for 48 h to stimulate the development of preantral follicles to antral

follicles, and the ovaries of the animals were removed after the animals were sacrificed.

Granulosa cell culture

The ovaries were cleared from the surrounding fat and oviduct, and punctured with 27-gauge needles in phenol red-free Dulbecco's Modified Eagle's medium (DMEM)/F12 medium (Invitrogen, Carlsbad, CA, USA), modified as described previously [27]. Granulosa cells were pelleted by centrifugation at 1000 rpm (4°C, 10 min) and resuspended in phenol red-free DMEM/F12 medium with 0.1% fatty acid-free bovine serum albumin, 1% fetal bovine serum, and 2 μg/mL bovine insulin. The follicular debris was then removed, and the granulosa cell suspensions were filtered through a cell strainer (BD Falcon, Bedford, MA, USA). The cells were plated at a concentration of 2.5×10^6 per well in a 6-well plate and were allowed to attach and grow to confluence for 1 day at 37°C, 5% CO_2, and 95% air. The cultured cells were then incubated in serum/phenol red-free medium (DMEM/F12 containing 0.1% lactalbumin enzymatic hydrolysate) overnight before the beginning of treatment. The cells were treated with different doses of testosterone (0.01, 0.1 or 1 μg/mL) or testosterone combined with $1,25D_3$ (0.1 μM) in 2 mL of serum/phenol red-free medium for 24 h. Granulosa cells were pretreated with an L-type calcium channel blocker nifedipine (NIF, 10 μM, Sigma, St. Louis, MO, USA) or an intracellular calcium chelator bis-(a-aminophe-noxy)-ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA-AM, 10 μM, Tocris, Minneapolis, MN, USA) for 30 min, pretreated with $1,25D_3$ for 15 min, and treated with one of three testosterone doses or vehicle for 24 h. At the end of the incubation period, the medium was collected, and the cells were lysed in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% Triton X-100, protease inhibitors and phosphatase inhibitors. A protein extract from the supernatant was used for western blot analysis.

Western blot

The protein content of the extracts was determined using the Bio-Rad Protein Assay Reagent. Equal amounts of the protein extract (15 μg) were mixed with loading buffer and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). After blocking for 1 h with 5% non-fat milk powder in Tris Buffered Saline (25 mM Tris, 135 mM NaCl and 2.5 mM KCl) with 0.05% Tween-20 (TBST), the membranes were incubated overnight with primary antibodies in TBST containing 5% non-fat milk powder and subsequently with horseradish peroxidase (HRP)-conjugated secondary antibody (Millipore, Billerica, MA, USA) in TBST with 5% non-fat milk powder at room temperature for 1 h. For experiments involving re-immunoblotting to different antibodies, the blots were stripped in 0.2 M glycine (pH 2.5) and 0.05% Tween-20 at 80°C for 20 min and then rinsed twice with 0.09 M boric acid (pH 7.4), 0.9% NaCl, and 0.05% Tween-20. The membranes were immunoblotted with different antibodies: aromatase (1:1000, Serotec, kidlington, Oxford, UK), phosphotyrosine (1:5000, Millipore, Billerica, MA, USA), Cx43 (1:1000, Novex, San Diego, CA, USA), estradiol receptor β (ERβ) (1:1000, GeneTex, San Antonio, Texas, USA), VDR (1:1000, GeneTex, San Antonio, Texas, USA), and β-actin (1:10000, Chemicon, Temecula, CA, USA). Immunoreactivity was detected by chemiluminescence autoradiography (ECL kit, Millipore, Billerica, MA, USA) in accordance with the manufacturer's instructions. The protein bands were quantified using the NIH image J Software.

Immunoprecipitation-western blot analysis

Granulosa cells were lysed in ice-cold lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% Triton X-100, protease inhibitors, and phosphatase inhibitors. The clarified lysate was immunoprecipitated at 4°C for 4 h in 300 μl of NP40 buffer (150 mM NaCl, 1% NP40, 50 mM Tris–HCl, pH 8.0, protease inhibitors, and phosphatase inhibitors) with anti-aromatase antibody (Serotec, kidlington, Oxford, UK) and protein G (GE Healthcare, Piscataway, NJ, USA). After washing, the immunoprecipitates were boiled, separated by 10% SDS-PAGE, and transferred to PVDF membranes. An Easyblot kit (GeneTex, San Antonio, Texas, USA) was used to decrease the interference caused by the heavy and light chains of the IgG. EasyBlocker was used as a blocking buffer to minimize the background caused by protein G contamination, and an EasyBlot anti-mouse IgG (HRP) was used that specifically reacts with the native form of mouse IgG and does not bind to the denatured form.

Aromatase activity assay

The enzyme aromatase is responsible for the synthesis of estradiol from testosterone. Hence, the effect of $1,25D_3$ on 17β-estradiol secretion by granulosa cells was used as an indicator of aromatase activity (modified by Zaher Merhi et al., 2014) [28]. To study the effect of $1,25D_3$ on the aromatase activity, granulosa cells were cultured in 24-well culture plates for 24 h to attach to the plate. After 24 h of culture, the cells were treated with testosterone or $1,25D_3$/testosterone (i.e. $1,25D_3$ plus testosterone), the medium was collected at the indicated times (10 min, 30 min, 1 h, 6 h and 24 h), and the 17β-estradiol level was measured. In order to exclude the earlier difference in 17β-estradiol secretion, the culture medium was removed after 18 h and replaced with fresh

medium supplemented with testosterone or $1,25D_3$/testosterone for another 6 h (18–24 h) treatment. The medium was collected for the measurement of 17β-estradiol concentrations at 18–24 h after the addition of the testosterone or $1,25D_3$/testosterone.

Radioimmunoassay

The cell culture medium was collected and stored at −80°C until the assay was performed. 17β-Estradiol levels were assayed using a Coat-A-Count Estradiol RIA kit (Siemens, Dublin, Ireland) according to the manufacturer's protocol. 1 mL of ^{125}I-labeled estradiol and 100 μL samples were incubated in anti-estradiol antibody-coated tubes for 3 h at room temperature. After decanting the mixture and washing the tubes, the radioactivity levels of the tubes were counted in a gamma counter. The counts are inversely related to the amount of 17β-estradiol present in the sample. The intra assay coefficients of variation for assays ranged between 3% and 16%, with a mean of 10.2%. The percentage cross-reactivity of this antiserum was 17α-estradiol: not detectable; estriol: 0.32%; estrone-β-D-glucuronide: 1.8%. The limit and highest of detection of the assay was 0–3600 pg/mL.

3-(4,5-dimethylthianol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

Cell viability was measured by a colorimetric MTT reduction assay performed as first described by Mosoman [29]. Each culture well was incubated in 0.5 mg/mL MTT (Sigma, St. Louis, MO, USA) culture medium followed by incubation for 4 h in 5% CO_2 at 37°C. The culture medium was then aspirated, and cells were lysed with DMSO. Quantitation of MTT reduction was assayed by measuring the absorbance at 570 nm (against the 630 nm reference) using an ELISA reader (BioTek, Winooski, VT, USA).

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA from granulosa cell cultures was isolated using a Total RNA Purification Kit (GeneMark, GMbiolab, Taichung, Taiwan) according to the manufacturer's instructions. RNA concentrations and purity were determined using a NanoDrop ND2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from 300 ng of total RNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA were used as templates in subsequent qPCR by an ABI 7500 PCR Detection System (Applied Biosystem, Foster City, CA, USA). The primer sequences for Cx43 used for PCR amplifications were as follows: 5′-TTG TTT CTG TCA CCA GTA AC-3′ (antisense) and 5′-GAT GAG GAA GGA AGA GAA GC-3′ (sense). GAPDH was used as the internal control: 5′-CCG CCT GCT TCA CCA CCT TCT-3′ (antisense) and 5′-GTC ATC ATC TCC GCC CCT TCC-3′ (sense). Each reaction contained the RT mixture, primers and SYBR Green Master Mix (Invitrogen, Carlsbad, CA, USA) and was carried out with the following profile: initial heating to 95°C for 10 min followed by 40 cycles of heating to 95°C for 15 s, incubation at 55°C for 30 s and incubation at 72°C for 90 s. Melting-curve analysis and PCR products were run on agarose gels with ethidium bromide staining. Cx43 mRNA levels were normalized to GAPDH and expressed as values relative to the control using the comparative threshold cycle method.

Immunocytochemistry

Granulosa cells were cultured on poly-L-lysine coated coverslips in 6-well plates. After 24 h of treatment, the granulosa cells were fixed in cold 4% paraformaldehyde for 20 min, rinsed three times with phosphate-buffered saline, and permeabilized with 0.1% Triton. Cells were blocked for 1 h with 5% goat serum and incubated for overnight at 4°C with rabbit anti-Cx43 (diluted 1:100, Novex, San Diego, CA, USA). The cells were then incubated for 1 h with FITC-conjugated goat anti-mouse IgG. Nuclei were stained with DAPI. Immunofluorescence images were captured using a confocal microscope (Zeiss LSM 700, Germany).

Statistical analysis

All biochemical data were analyzed with Student's t-test or a one-way analysis of variance (one-way ANOVA). Specific comparisons between any experimental group and a common control group were made using Dunnett's *t*-test. Comparisons between two experimental groups were made using the Student-Newman-Keuls method. Statistical significance was evaluated at the levels of $p < 0.05$, $p < 0.005$, and $p < 0.001$.

Results

$1,25D_3$ attenuated testosterone-induced aromatase expression and increased 17β-estradiol secretion

To examine whether $1,25D_3$ and testosterone regulate aromatase expression and 17β-estradiol secretion in granulosa cells, we treated cells with 0.1 or 1 μg/mL testosterone or 0.1 μM $1,25D_3$ plus 1 μg/mL testosterone for 24 h. Western blot analysis revealed that both doses of testosterone increased the aromatase levels ($p < 0.005$; Figure 1A) compared to vehicle-treated cells, and addition of 0.1 μM $1,25D_3$ resulted in a significant decrease of the 1 μg/mL testosterone-induced aromatase levels ($p < 0.005$; Figure 1A). Additionally, treatment with $1,25D_3$ alone attenuated aromatase expression compared to vehicle treatment ($p < 0.05$; Figure 1A). We also examined 17β-estradiol secretion using a radioimmunoassay and found that both testosterone-treated

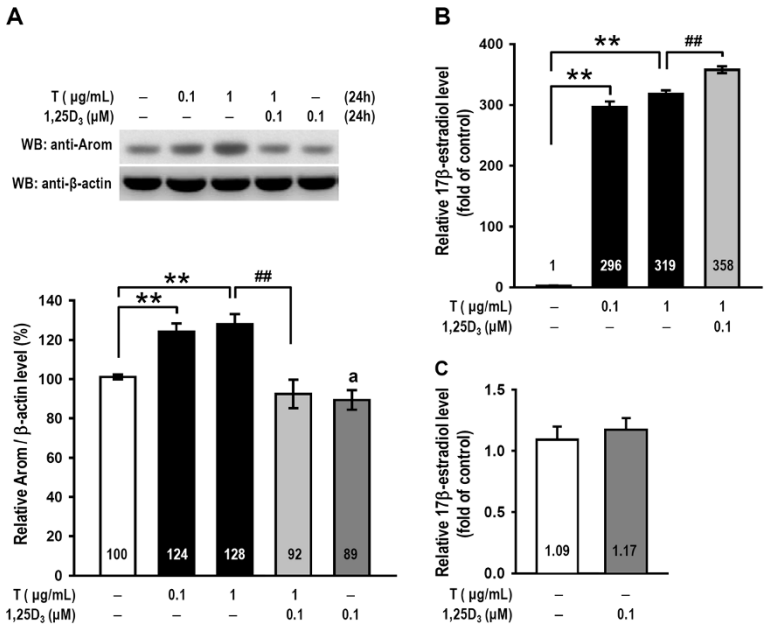


Figure 1 $1,25D_3$ attenuated testosterone-induced aromatase expression but improved 17β-estradiol secretions. Ovarian granulosa cells from rats were pretreated with $1,25D_3$ (0.1 μM) for 15 min and subsequently treated with 0.1 μg/mL or 1 μg/mL testosterone (denoted by T) or vehicle (denoted by –) for 24 h. **(A)** Cells were lysed for protein extraction, and cell lysates were subjected to western blot analysis for aromatase protein detection. The level of aromatase (denoted by Arom) expression was estimated by densitometric analyses after normalization with the β-actin signal (n = 5–6 in each group). **(B and C)** 17β-Estradiol secretions from the granulosa cells were collected from the medium and assessed over the course of a 24-h period of drug treatments. 17β-Estradiol release data were expressed as the fold-change relative to the vehicle-treated control (n = 4–5 in each group). The values represent the means ± SEM. **, $p < 0.005$ for comparison with the vehicle-treated group; ##, $p < 0.005$ for comparison of testosterone/$1,25D_3$ and testosterone alone; a, $p < 0.05$ for comparison of $1,25D_3$ alone and vehicle-treated group. The values listed within column indicate the means.

groups clearly experienced enhanced 17β-estradiol secretion ($p < 0.005$; Figure 1B). The $1,25D_3$/testosterone group exhibited significantly increased 17β-estradiol secretion relative to the group without $1,25D_3$ treatment ($p < 0.005$). Treatment of cells with 0.1 μM $1,25D_3$ alone for 24 h did not result in a significant increase in 17β-estradiol secretion ($p > 0.05$; Figure 1C). These results indicate that $1,25D_3$ has the potential to improve testosterone-induced 17β-estradiol secretion but causes a decrease in testosterone-induced aromatase expression.

$1,25D_3$ significantly increased aromatase tyrosine phosphorylation

We evaluated whether the phosphorylation of aromatase was regulated by testosterone and $1,25D_3$ using immunoprecipitation-western blot analysis. We determined that the $1,25D_3$/testosterone group exhibited significantly increased the level of aromatase tyrosine phosphorylation at 24 h relative to the testosterone group ($p < 0.05$; Figure 2A, left), and $1,25D_3$ alone also significantly increased the level of aromatase tyrosine phosphorylation relative to the control group ($p < 0.05$; Figure 2A, right). The left panel in Figure 2B is a positive control that was both immunoprecipitated and immunoblotted with an anti-aromatase antibody, and the right panel is a negative control for IgG. The lysate of the granulosa cells treated with $1,25D_3$/testosterone showed a significant band attributed to immunoprecipitated aromatase phosphotyrosine and a weak band for IgG.

We next investigated the time course of the phosphotyrosine response to testosterone and to $1,25D_3$/testosterone. Our data demonstrate that testosterone treatment induced aromatase tyrosine phosphorylation at 10 min, 30 min and 1 h, whereas $1,25D_3$ improved the effect of testosterone at 30 min and increased the duration of the effect to 6 h ($p < 0.05$; Figure 2C) and 24 h (Figure 2A). We also collected the medium for the 17β-estradiol assay and determined that testosterone-induced 17β-estradiol secretion increased at 30 min, 1 h ($p < 0.05$) and 6 h (n = 5; $p < 0.001$; Figure 2D). There was no difference in 17β-estradiol secretion between the testosterone-treated groups and the $1,25D_3$/testosterone-treated groups at up to 6 h ($p > 0.05$; Figure 2D). We also collected the medium 18–24 h after drug treatment and found that the $1,25D_3$/testosterone-treated group significantly increased 17β-estradiol secretion ($p < 0.05$; Figure 2E). These studies demonstrated that $1,25D_3$ increased aromatase tyrosine phosphorylation and 17β-estradiol secretion.

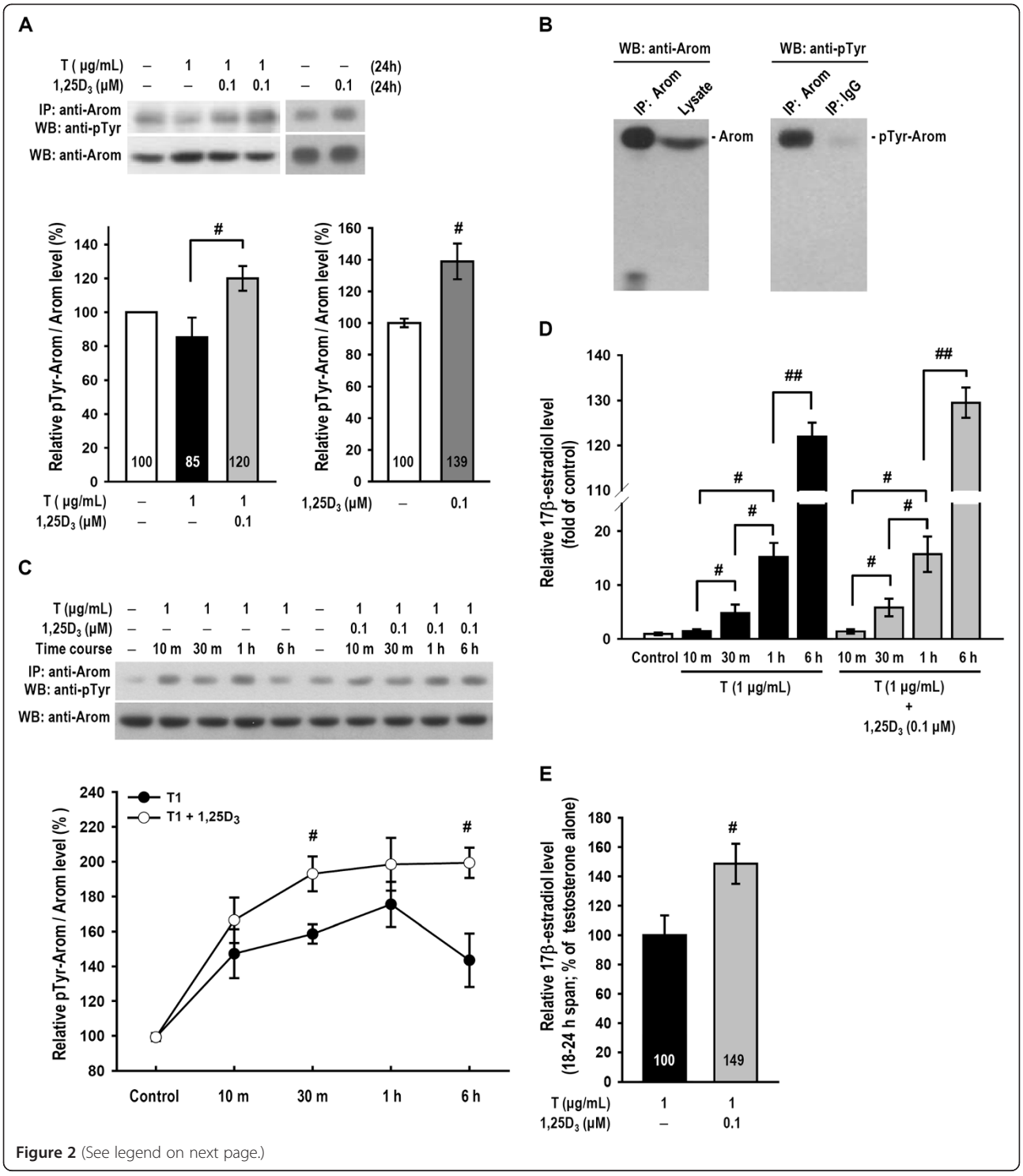


Figure 2 (See legend on next page.)

(See figure on previous page.)

Figure 2 1,25D$_3$ enhanced testosterone-induced aromatase tyrosine phosphorylation and 17β-estradiol secretion during 18–24 h. (A) Rat ovarian granulosa cells were treated with vehicle, testosterone, testosterone plus 1,25D$_3$ or 1,25D$_3$ alone for 24 h (n = 4–5 in each group). Aromatase was immunoprecipitated before being subjected to SDS-PAGE for resolution. The resultant blot was probed using both phosphotyrosine and aromatase antibodies. The expression of aromatase tyrosine phosphorylation (denoted by pTyr-Arom) was normalized to the corresponding aromatase expression and is represented as a percentage relative to the vehicle-treated control. **(B)** The left panel is a positive control that was both immunoprecipitated and immunoblotted with an anti-aromatase antibody. The lysate was also immunoprecipitated with the anti-aromatase antibody or IgG and immunoblotted with an anti-phosphotyrosine antibody, as shown in the right panel. A weak band in the IgG lane is as a negative control. **(C)** Cells were treated with testosterone alone or testosterone plus 1,25D$_3$ as indicated for various time periods (n = 4–5 in each group). Each pTyr-Arom level was normalized using the corresponding aromatase level and is represented as a percentage relative to the vehicle-treated control. **(D)** After treatment for various time periods as indicated, the 17β-estradiol production levels were measured by radioimmunoassay. **(E)** After treatment with testosterone alone or testosterone plus 1,25D$_3$ for 18 h, the culture medium was removed and then replaced with fresh medium. Cells were continuously treated for another 6 h (18–24 h span). After the final 6 h, medium was collected and the 17β-estradiol secretion levels were measured by radioimmunoassay (n = 6 in each group). The values represent the means ± SEM. #, $p < 0.05$; ##, $p < 0.005$ for comparison of the two treatment groups. The values listed within column indicate the means.

1,25D$_3$ and testosterone-induced 17β-estradiol production was mediated by calcium

One possible mechanism for 1,25D$_3$/testosterone modulation of 17β-estradiol production is through increasing calcium concentrations. We studied whether NIF or BAPTA-AM could inhibit 1,25D$_3$/testosterone-regulated 17β-estradiol secretion. A radioimmunoassay of 17β-estradiol production is presented in Figure 3. Statistical analysis revealed that testosterone doses (0.01, 0.1 and 1 μg/mL) increased 17β-estradiol production ($p < 0.001$ and $p < 0.005$), and 1,25D$_3$ (0.1 μM) increased high-dose (1 μg/mL) testosterone-induced 17β-estradiol secretion ($p < 0.05$; Figure 3A). Our data demonstrate that pre-treatment with NIF reduced 1,25D$_3$ and testosterone

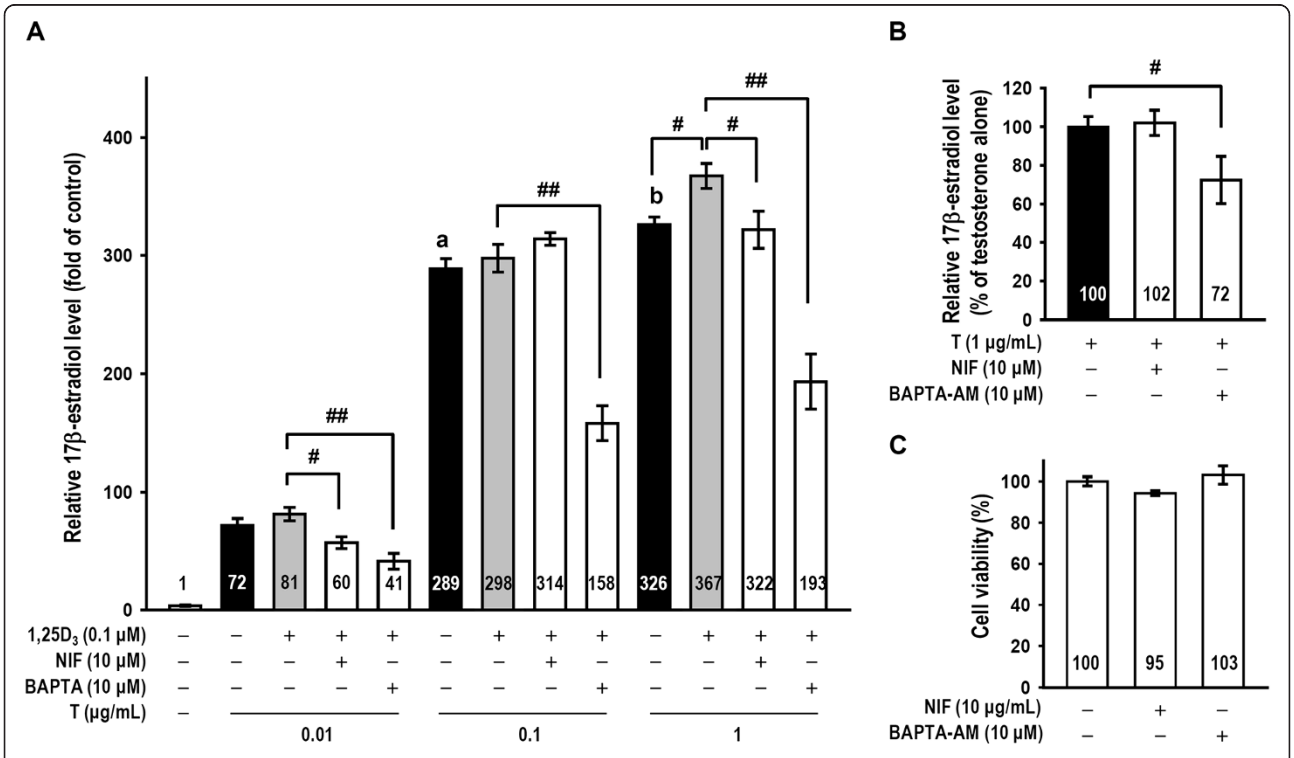


Figure 3 1,25D$_3$ and testosterone-induced 17β-estradiol secretion was mediated by calcium accumulation. (A) Granulosa cells were divided into vehicle, testosterone, testosterone plus 1,25D$_3$, L-type calcium channel blocker NIF (10 μM) or intracellular calcium chelator BAPTA-AM (10 μM) pre-treatment groups (n = 4–5 in each group). The individual levels of 17β-estradiol were also assayed using a radioimmunoassay. **(B)** Testosterone-induced 17β-estradiol released by granulosa cells cultured with or without NIF or BAPTA-AM in the absence of 1,25D$_3$ was measured (n = 9 in each group). **(C)** After NIF or BAPTA-AM treatment for 24 h, cells were subjected to the MTT reduction assay to measure cell viability. MTT reduction data are expressed as the percentage of values relative to the control group. The measurements were performed in triplicate, and each cytotoxicity experiment was repeated four times. The values represent the means ± SEM. #, $p < 0.05$; ##, $p < 0.005$ for comparison of two drug groups; a, $p < 0.001$ for comparison of 0.1 μg/mL and 0.01 μg/mL testosterone; b, $p < 0.05$ for comparison of 1 μg/mL and 0.1 μg/mL testosterone. The values listed within column indicate the means.

(0.01 and 1 μg/mL)-enhanced 17β-estradiol secretion in granulosa cells ($p < 0.05$; Figure 3A) but not testosterone (1 μg/mL)-induced 17β-estradiol secretion (Figure 3B). BAPTA-AM reduced 17β-estradiol secretion induced by $1,25D_3$/testosterone (0.01, 0.1 and 1 μg/mL) ($p < 0.001$; Figure 3A) and by testosterone (1 μg/mL) alone ($p < 0.05$; Figure 3B). We also monitored cell viability using an MTT reduction assay. The viability of cultured cells exposed to NIF (10 μM) or BAPTA-AM (10 μM) did not significant differ from the control group ($p > 0.05$; Figure 3C). Thus, these data revealed that chelation of intercellular calcium with BAPTA-AM resulted in suppression of both testosterone- and $1,25D_3$/testosterone-modulated 17β-estradiol secretion, while calcium from the L-type calcium channel was involved in only $1,25D_3$/testosterone-modulated 17β-estradiol secretion in cultured granulosa cells.

The effects of testosterone and $1,25D_3$ on ERβ or VDR expression

We examined the expression levels of ERβ and VDR in granulosa cells to determine if they were altered by testosterone and $1,25D_3$. Western blot analysis revealed that granulosa cells treated with testosterone (0.1 and 1 μg/mL) exhibited markedly increased ERβ expression levels ($p < 0.005$; Figure 4A) and that 1 μg/mL testosterone treatment increased ERβ expression more than treatment with 0.1 μg/mL testosterone ($p < 0.05$). However, the two doses of testosterone did not alter VDR levels ($p > 0.05$; Figure 4B). Additionally, pre-treatment with $1,25D_3$ did not alter testosterone-induced ERβ expression and did not influence VDR expression (n = 3–4, $p > 0.05$; Figure 4A, B). Thus, testosterone alone affected ERβ expression, but $1,25D_3$ pre-treatment had no influence on ERβ or VDR in cultured granulosa cells.

$1,25D_3$ reversed testosterone-induced down-regulation of Cx43 in granulosa cells

Finally, to examine whether $1,25D_3$ can affect the testosterone-induced down-regulation of Cx43, granulosa cells were treated with testosterone in the presence or absence of 0.1 μM $1,25D_3$. We determined the cellular localization of Cx43 using immunofluorescence and observed its expression as green fluorescent plaques (Figure 5A). Cx43 protein expression was markedly decreased when cells were treated with a high dose of testosterone (1 μg/mL; $p < 0.005$), but this effect was reversed by pre-treatment with $1,25D_3$ ($p < 0.05$; Figure 5A, B). We performed a real-time PCR analysis and found that cultured cells pre-treated with $1,25D_3$ also reversed testosterone (1 μg/mL)-induced down-regulation of Cx43 ($p < 0.05$; Figure 5C). These results indicated that $1,25D_3$ abolishes the inhibitory effect of testosterone on Cx43 expression in granulosa cells.

Discussion

Testosterone can induce concentration-dependent physiological and pathological effects. Physiologic levels of testosterone are converted into sufficient 17β-estradiol to improve follicular development, and the pathological

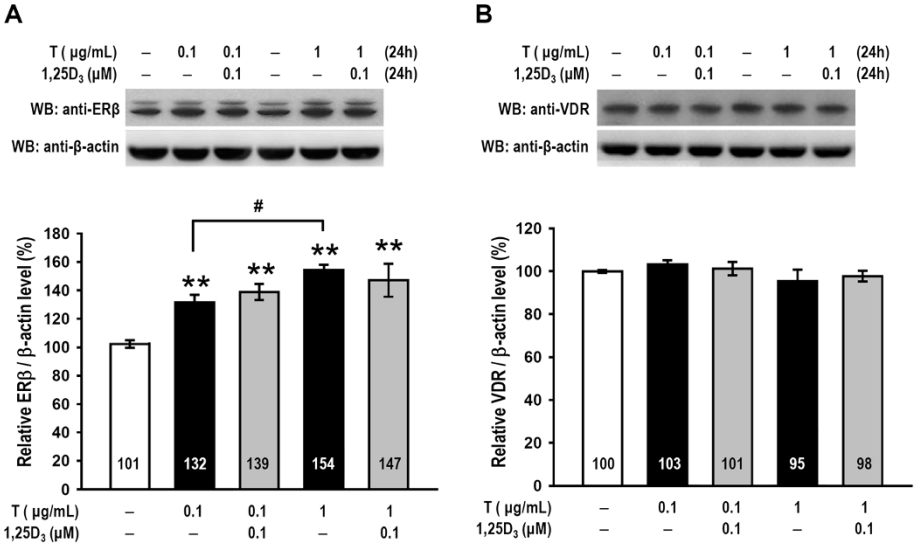


Figure 4 Testosterone, but not $1,25D_3$, enhanced ERβ expression; testosterone and $1,25D_3$ had no effect on VDR expression. Granulosa cells were pretreated with $1,25D_3$ for 15 min and subsequently treated with one of two testosterone doses or vehicle for 24 h (n = 4–5 in each group). The granulosa cell lysates were subjected to western blot analysis for **(A)** ERβ and **(B)** VDR expression levels. The levels of ERβ and VDR expression were quantified using densitometry and normalized to β-actin. The values represent the means ± SEM. **, $p < 0.005$ for comparison with the vehicle-treated group. #, $p < 0.05$ for comparison of two drug groups. The values listed within column indicate the means.

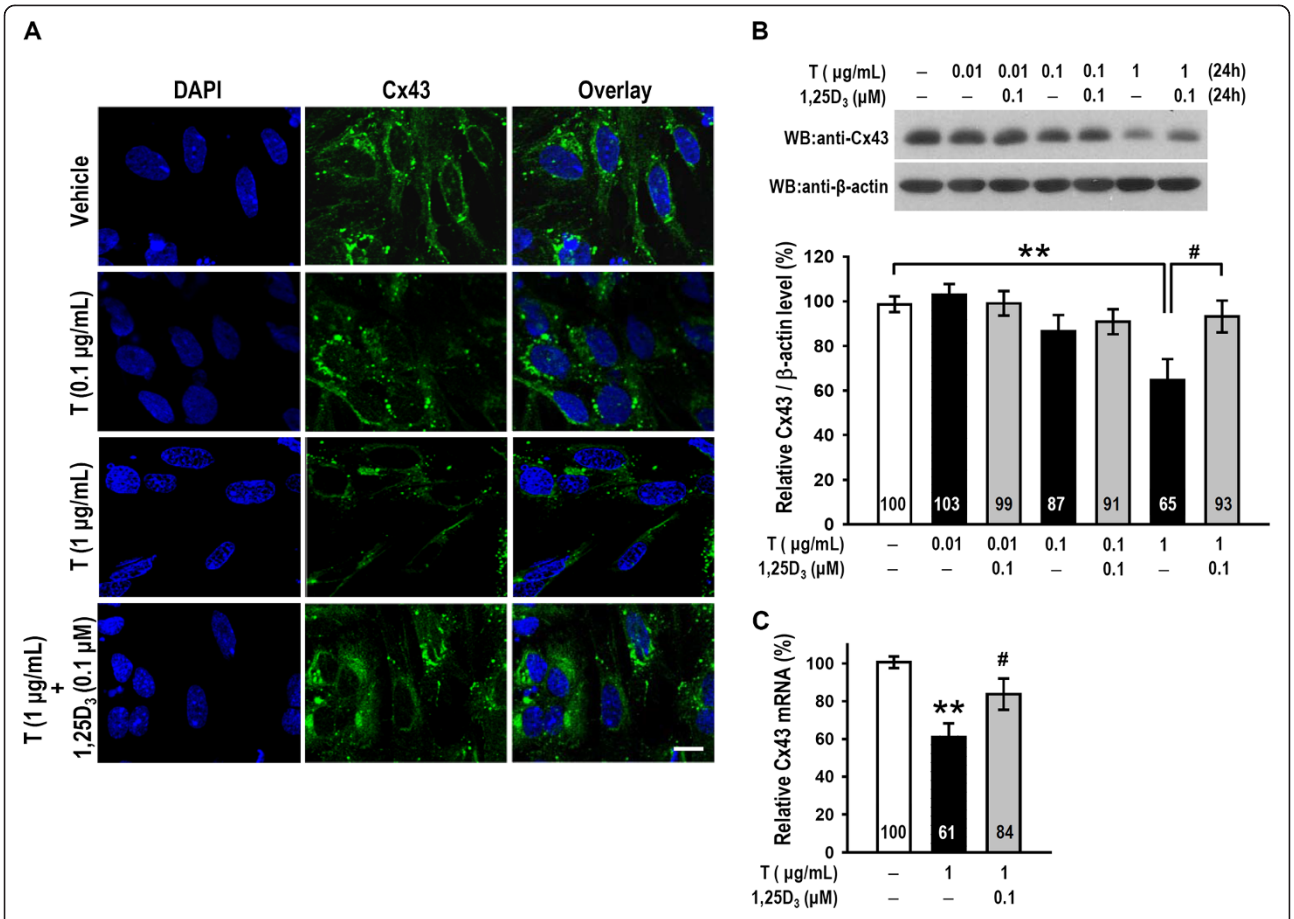


Figure 5 Testosterone-induced down-regulation of Cx43 in granulosa cells was reversed by treatment with $1,25D_3$. (A) Granulosa cells were treated for 24 h with vehicle, testosterone (0.1 or 1 μg/mL) or testosterone (1 μg/mL) plus $1,25D_3$ (0.1 μM) and fixed in cold paraformaldehyde solution. Cell nuclei were stained with DAPI (left column), and Cx43 levels (green) were examined by confocal microscopy (middle column). The overlay is shown in the right column. **(B, C)** Granulosa cells were treated with 0.01, 0.1, or 1 μg/mL testosterone for 24 h in the presence of vehicle control or 0.1 μM $1,25D_3$. The levels of Cx43 protein and Cx43 mRNA were examined by western blot and qRT-PCR analysis, respectively. The results are expressed as the means ± SEM (n = 4–6 in each group). **, $p < 0.005$ for comparison with the vehicle-treated group. #, $p < 0.05$ for comparison of two drug groups. The values listed within column indicate the means. Scale bars, 10 μm.

effects of testosterone may be due to induced hyperandrogenism and arrested follicular development. Androgen concentrations in the follicular fluid (FF) are higher in small follicles than in large follicles, and the androgen concentration in PCOS FF is higher than in FF from healthy women [30,31]. In the present study, we have demonstrated the dose dependence of testosterone-induced aromatase expression (Figure 1A) and 17β-estradiol secretion (Figures 1B and 3A). These results are consistent with a previous report demonstrating that testosterone increases aromatase mRNA levels [2]. Testosterone increases the levels of aromatase mRNA promoter 1.1- and 1.5-derived transcripts at 1 μM but only increases the promoter 2-derived transcript at the highest (100 μM) dose [9]. Interestingly, we found that $1,25D_3$ reduced testosterone-induced aromatase expression but stimulated 17β-estradiol secretion (Figure 1A, B). $1,25D_3$ appears to modulate aromatase expression in the estrogenesis of granulosa cells and has been shown to exert tissue-specific effects on aromatase expression by various promoters [32]. For example, $1,25D_3$ increases aromatase expression in placental cells and osteoblasts but down-regulates aromatase expression in breast cancer cells [33-35]. Lundqvist et al. also demonstrated that a $1,25D_3$ analogue reduces aromatase expression by promoting dissociation of the co-modulator Williams syndrome transcription factor from the *cyp19a1* promoter in breast cancer cells [36]. In addition, competition for shared co-regulators between VDR and AR is one possible explanation for the suppressive effect of $1,25D_3$/VDR signals on AR transcriptional activity [37]. In our study, we found that treatment with $1,25D_3$ alone reduced aromatase expression but did not alter 17β-estradiol secretion (Figure 1A, C). Thus, $1,25D_3$ exhibits a synergist effect in attenuating testosterone-induced aromatase expression.

Recently, aromatase activity was shown to mediate post-translational modifications. Changes in aromatase activity

often reflect differential protein expression arising from a slow rate of mRNA transcription. The phosphorylation of aromatase rapidly regulates estradiol production. For example, studies of murine aromatase suggest that serine 118 [38] or tyrosine 361 [20] can be phosphorylated and affect aromatase stability or activity. Specifically, increases in aromatase activity and estradiol secretion are regulated by c-Src kinase-catalysed tyrosine phosphorylation [20] and inhibits in aromatase activity by protein tyrosine phosphatase 1B in breast cancer cells [39]. In our study, although $1,25D_3$ inhibited aromatase protein expression, we also found that it can regulate tyrosine phosphorylation and change the activity of aromatase to improve 17β-estradiol secretion at longer times. We demonstrated that $1,25D_3$ treatment led to a significantly increased aromatase tyrosine phosphorylation level at 30 min, 6 h (Figure 2C) and 24 h (Figure 2A, left) compared with testosterone alone (Figure 2A, C). $1,25D_3$ also increased the level of aromatase tyrosine phosphorylation without testosterone at 24 h (Figure 2A, right). No difference in 17β-estradiol concentration between the testosterone and $1,25D_3$/testosterone groups was observed within the first 6 h (Figure 2D), but $1,25D_3$ markedly increased 17β-estradiol secretion at 18–24 h (Figure 2E). These results might suggest that a sustained, $1,25D_3$-induced increase in aromatase tyrosine phosphorylation maintains the effect of testosterone on 17β-estradiol secretion at 18–24 h. Thus, the 13% increase in 17β-estradiol production might arise at later times during the 24 h treatment with $1,25D_3$. Because VDRs are located in the largest follicles, the 13% increase in 17β-estradiol from $1,25D_3$/testosterone treatment might assist growth of the largest follicles. $1,25D_3$ might in this way inhibit aromatase expression and prevent aromatase excess syndrome, and increased aromatase tyrosine phosphorylation may rapidly regulate 17β-estradiol in the appropriate time frame.

$1,25D_3$ is also known to promote calcium absorption from intestinal cells [40]; however, there was no clear evidence suggesting that $1,25D_3$ increased calcium accumulation in granulose cells. Two different calcium channels (T-type and L-type) are involved in steroidogenesis in granulose cells [41]. In this study, we observed that an L-type calcium channel blocker reduced 17β-estradiol secretion under $1,25D_3$/testosterone (0.01 or 1 μg/mL) treatment (Figure 3A). $1,25D_3$/testosterone-induced 17β-estradiol secretion was also significantly reduced by an intracellular calcium chelator. Additionally, NIF (10 μM) or BAPTA-AM (10 μM) alone did not cause cell toxicity (Figure 3C), and this dose of BAPTA-AM also reduces testosterone-induced 17β-estradiol secretion without $1,25D_3$ (Figure 3B). These results are similar to a report that implicated a calcium-dependent pathway in mediation of gonadotropin-induced steroidogenesis in the ovary [42]. Weitzel et al. demonstrated that calcium signals are critical in the inhibition of low-density lipoprotein receptor-1-mediated estradiol production in murine granulosa cells [43]. Our data is the first to illustrate that stimulation of 17β-estradiol production by $1,25D_3$ and testosterone is mediated by the L-type calcium channel and intracellular calcium levels (Figure 3).

Several effects of 17β-estradiol have been shown to be important for follicular development and ovarian function, including the regulation of ER levels, stimulation of DNA synthesis, cell proliferation and regulation of atresia in ovarian follicles [44]. ERβ is the important ER member expressed in growing granulosa cells and the mature follicle in rodent ovaries and is critical to granulosa cell proliferation and differentiation [45]. We have demonstrated that testosterone-treated granulosa cells exhibited significantly increased ERβ levels and that $1,25D_3$ did not alter testosterone-induced ERβ expression (Figure 4A). This result is similar to the finding that the expression levels of ERβ mRNA in the ovaries of VDR-null mutant and wild-type mice are the same [16]. Additionally, both testosterone and $1,25D_3$ had no effect on VDR expression (Figure 4B). These results might suggest that testosterone or $1,25D_3$/testosterone increases ERβ levels but not VDR levels.

Intercellular and intracellular endocrine regulatory mechanisms may be critical for follicle growth, dominant follicle selection, and follicle atresia. As ovarian follicles grow from the small to the large antral stage, granulosa cells provide 17β-estradiol by aromatase activation for dominant follicle development [46]. In contrast, other small follicles undergo atresia via apoptosis [47]. AR is expressed in primordial follicles, advanced-stage follicles and primary follicles [5], and testosterone stimulates the early stages of follicle growth, inhibits preovulatory follicle apoptosis and limits follicle size [6]. Thus, the observation that many small follicles grow in PCOS may be explained by excess testosterone. Alternatively, $1,25D_3$ might inhibit the testosterone-stimulated early stages of follicular growth because $1,25D_3$ is known to inhibit the proliferation and induce the differentiation of a variety of cells [48], and VDR is expressed in the granulosa cells of largest follicle [49]. It is possible that $1,25D_3$ paired with testosterone preferentially improves the growth of larger follicles. Hence, the effect of $1,25D_3$ and testosterone on aromatase in granulosa cells might indicate a plausible treatment option for PCOS. Several reports have also shown that $1,25D_3$ regulates antimüllerian hormone (AMH) signalling, follicle-stimulating hormone sensitivity, and progesterone production in human granulosa cells, and decreases the abnormally elevated AMH levels in $1,25D_3$-deficient women with PCOS, indicating a critical role for $1,25D_3$ in follicular development [50].

Intercellular communication via gap junctions is vitally important for granulosa cell differentiation and oocyte

growth [22]. In this study, western blot analysis, qRT-PCR and immunostaining showed that high dose (1 μg/mL) of testosterone decreased Cx43 protein expression in granulosa cells, and this decrease was reversed by co-treatment with 0.1 μM $1,25D_3$ (Figure 5). These results are similar to the finding that excess DHT down-regulates Cx43 in granulosa cells [25]. The correlation between abnormal androgen concentrations and Cx43 expression might contribute to the pathogenesis of PCOS, and $1,25D_3$ might prevent Cx43 down-regulation.

Conclusions

We determined that testosterone treatment increased aromatase expression and increased 17β-estradiol secretion in granulosa cells. $1,25D_3$ decreased testosterone-induced aromatase expression but significantly increased aromatase phosphotyrosine levels and lengthened the duration of testosterone-induced 17β-estradiol secretion in rat granulosa cells to 18–24 h. We also determined that $1,25D_3$/testosterone-induced 17β-estradiol secretion is dependent on changes in calcium levels. Additionally, testosterone increased ERβ expression, but neither $1,25D_3$ nor testosterone mediated VDR expression. Finally, a high dose of testosterone reduced Cx43 expression, and $1,25D_3$ abolished testosterone-reduced Cx43 levels.

Abbreviations

AMH: Antimüllerian hormone; AR: Androgen receptors; BAPTA-AM: bis-(a-aminophe-noxy)-ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl)-ester; Cx43: Connexin 43; $1,25D_3$: 1,25-dihydroxyvitamin D_3; DHT: Dihydrotestosterone; ERβ: Estradiol receptor β; FF: Follicular fluid; FSH: Follicle-stimulating hormone; NIF: Nifedipine; PCOS: Polycystic ovary syndrome; VDR: 1,25-dihydroxyvitamin D_3 receptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CTL conceived and performed experiments, conducted statistical analysis and drafted the manuscript. JYW and KYC contributed intellectual input toward the study's design and reviewed the manuscript. MIH supervised and contributed to data interpretation. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Taiwan National Science Council Grant NSC 102-2629-B-038-001, Taipei Medical University of Taiwan (TMU98-AE1-B01), and Hsin Sheng College of Medical Care and Management of Taiwan (HSC-102-010). English language correction of this article has been accomplished by language editing services, ELSEVIER.

Author details

[1]Department of Nursing, Hsin Sheng College of Medical Care and Management, Taoyuan, Taiwan. [2]Department of Neurology, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan. [3]General Education Center, National Taipei University of Nursing and Health Sciences, Taipei, Taiwan. [4]Department of Obstetrics and Gynecology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan.

Received: 8 May 2014 Accepted: 13 September 2014
Published: 20 September 2014

References

1. Ryan KJ: **Biochemistry of aromatase: significance to female reproductive physiology.** *Cancer Res* 1982, **42:**3342s–3344s.
2. Luo W, Wiltbank MC: **Distinct regulation by steroids of messenger RNAs for FSHR and CYP19A1 in bovine granulosa cells.** *Biol Reprod* 2006, **75:**217–225.
3. Belin F, Goudet G, Duchamp G, Gerard N: **Intrafollicular concentrations of steroids and steroidogenic enzymes in relation to follicular development in the mare.** *Biol Reprod* 2000, **62:**1335–1343.
4. Stocco C: **Aromatase expression in the ovary: hormonal and molecular regulation.** *Steroids* 2008, **73:**473–487.
5. Otala M, Makinen S, Tuuri T, Sjoberg J, Pentikainen V, Matikainen T, Dunkel L: **Effects of testosterone, dihydrotestosterone, and 17beta-estradiol on human ovarian tissue survival in culture.** *Fertil Steril* 2004, **82**(Suppl 3)**:**1077–1085.
6. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA: **Androgens stimulate early stages of follicular growth in the primate ovary.** *J Clin Invest* 1998, **101:**2622–2629.
7. Murray AA, Gosden RG, Allison V, Spears N: **Effect of androgens on the development of mouse follicles growing in vitro.** *J Reprod Fertil* 1998, **113:**27–33.
8. Hu YC, Wang PH, Yeh S, Wang RS, Xie C, Xu Q, Zhou X, Chao HT, Tsai MY, Chang C: **Subfertility and defective folliculogenesis in female mice lacking androgen receptor.** *Proc Natl Acad Sci U S A* 2004, **101:**11209–11214.
9. Hamel M, Vanselow J, Nicola ES, Price CA: **Androstenedione increases cytochrome P450 aromatase messenger ribonucleic acid transcripts in nonluteinizing bovine granulosa cells.** *Mol Reprod Dev* 2005, **70:**175–183.
10. Wu YG, Bennett J, Talla D, Stocco C: **Testosterone, not 5alpha-dihydrotestosterone, stimulates LRH-1 leading to FSH-independent expression of Cyp19 and P450scc in granulosa cells.** *Mol Endocrinol* 2011, **25:**656–668.
11. Hsu MI, Liou TH, Chou SY, Chang CY, Hsu CS: **Diagnostic criteria for polycystic ovary syndrome in Taiwanese Chinese women: comparison between Rotterdam 2003 and NIH 1990.** *Fertil Steril* 2007, **88:**727–729.
12. Wehr E, Pilz S, Schweighofer N, Giuliani A, Kopera D, Pieber TR, Obermayer-Pietsch B: **Association of hypovitaminosis D with metabolic disturbances in polycystic ovary syndrome.** *Eur J Endocrinol* 2009, **161:**575–582.
13. Wehr E, Trummer O, Giuliani A, Gruber HJ, Pieber TR, Obermayer-Pietsch B: **Vitamin D-associated polymorphisms are related to insulin resistance and vitamin D deficiency in polycystic ovary syndrome.** *Eur J Endocrinol* 2011, **164:**741–749.
14. Lerchbaum E, Obermayer-Pietsch B: **Vitamin D and fertility: a systematic review.** *Eur J Endocrinol* 2012, **166:**765–778.
15. Johnson JA, Grande JP, Roche PC, Kumar R: **Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D3 receptor in rat reproductive tissues.** *Histochem Cell Biol* 1996, **105:**7–15.
16. Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S, Seino Y: **Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads.** *Endocrinology* 2000, **141:**1317–1324.
17. Boland R, Buitrago C, De Boland AR: **Modulation of tyrosine phosphorylation signalling pathways by 1alpha,25(OH)2-vitamin D3.** *Trends Endocrinol Metab* 2005, **16:**280–287.
18. Buitrago C, Vazquez G, De Boland AR, Boland RL: **Activation of Src kinase in skeletal muscle cells by 1, 1,25-(OH(2))-vitamin D(3) correlates with tyrosine phosphorylation of the vitamin D receptor (VDR) and VDR-Src interaction.** *J Cell Biochem* 2000, **79:**274–281.
19. Buitrago C, Vazquez G, De Boland AR, Boland R: **The vitamin D receptor mediates rapid changes in muscle protein tyrosine phosphorylation induced by 1,25(OH)(2)D(3).** *Biochem Biophys Res Commun* 2001, **289:**1150–1156.
20. Catalano S, Barone I, Giordano C, Rizza P, Qi H, Gu G, Malivindi R, Bonofiglio D, Ando S: **Rapid estradiol/ERalpha signaling enhances aromatase enzymatic activity in breast cancer cells.** *Mol Endocrinol* 2009, **23:**1634–1645.
21. Cheng J, Watkins SC, Walker WH: **Testosterone activates mitogen-activated protein kinase via Src kinase and the epidermal growth factor receptor in sertoli cells.** *Endocrinology* 2007, **148:**2066–2074.
22. Kidder GM, Mhawi AA: **Gap junctions and ovarian folliculogenesis.** *Reproduction* 2002, **123:**613–620.
23. Teilmann SC: **Differential expression and localisation of connexin-37 and connexin-43 in follicles of different stages in the 4-week-old mouse ovary.** *Mol Cell Endocrinol* 2005, **234:**27–35.
24. Ackert CL, Gittens JE, O'Brien MJ, Eppig JJ, Kidder GM: **Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse.** *Dev Biol* 2001, **233:**258–270.

25. Wu CH, Yang JG, Yang JJ, Lin YM, Tsai HD, Lin CY, Kuo PL: **Androgen excess down-regulates connexin43 in a human granulosa cell line.** *Fertil Steril* 2010, **94:**2938–2941.
26. Clairmont A, Tessman D, Stock A, Nicolai S, Stahl W, Sies H: **Induction of gap junctional intercellular communication by vitamin D in human skin fibroblasts is dependent on the nuclear Induction of gap junctional intercellular communication by vitamin D in human skin fibroblasts is dependent on the nuclear vitamin D receptor.** *Carcinogenesis* 1996, **17:**1389–1391.
27. Ke FC, Fang SH, Lee MT, Sheu SY, Lai SY, Chen YJ, Huang FL, Wang PS, Stocco DM, Hwang JJ: **Lindane, a gap junction blocker, suppresses FSH and transforming growth factor beta1-induced connexin43 gap junction formation and steroidogenesis in rat granulosa cells.** *J Endocrinol* 2005, **184:**555–566.
28. Merhi Z, Doswell A, Krebs K, Cipolla M: **Vitamin D alters genes involved in follicular development and steroidogenesis in human cumulus granulosa cells.** *J Clin Endocrinol Metab* 2014, **99:**E1137–1145.
29. Mosmann T: **Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.** *J Immunol Methods* 1983, **65:**55–63.
30. Teissier MP, Chable H, Paulhac S, Aubard Y: **Comparison of follicle steroidogenesis from normal and polycystic ovaries in women undergoing IVF: relationship between steroid concentrations, follicle size, oocyte quality and fecundability.** *Hum Reprod* 2000, **15:**2471–2477.
31. Eilso Nielsen M, Rasmussen IA, Fukuda M, Westergaard LG, Yding Andersen C: **Concentrations of anti-Mullerian hormone in fluid from small human antral follicles show a negative correlation with CYP19 mRNA expression in the corresponding granulosa cells.** *Mol Hum Reprod* 2010, **16:**637–643.
32. Lundqvist J, Norlin M, Wikvall K: **1alpha,25-Dihydroxyvitamin D3 exerts tissue-specific effects on estrogen and androgen metabolism.** *Biochim Biophys Acta* 1811, **2011:**263–270.
33. Krishnan AV, Swami S, Peng L, Wang J, Moreno J, Feldman D: **Tissue-selective regulation of aromatase expression by calcitriol: implications for breast cancer therapy.** *Endocrinology* 2010, **151:**32–42.
34. Enjuanes A, Garcia-Giralt N, Supervia A, Nogues X, Ruiz-Gaspa S, Bustamante M, Mellibovsky L, Grinberg D, Balcells S, Diez-Perez A: **Functional analysis of the I.3, I.6, pII and I.4 promoters of CYP19 (aromatase) gene in human osteoblasts and their role in vitamin D and dexamethasone stimulation.** *Eur J Endocrinol* 2005, **153:**981–988.
35. Barrera D, Avila E, Hernandez G, Halhali A, Biruete B, Larrea F, Diaz L: **Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol.** *J Steroid Biochem Mol Biol* 2007, **103:**529–532.
36. Lundqvist J, Hansen SK, Lykkesfeldt AE: **Vitamin D analog EB1089 inhibits aromatase expression by dissociation of comodulator WSTF from the CYP19A1 promoter-a new regulatory pathway for aromatase.** *Biochim Biophys Acta* 1833, **2013:**40–47.
37. Ting HJ, Bao BY, Hsu CL, Lee YF: **Androgen-receptor coregulators mediate the suppressive effect of androgen signals on vitamin D receptor activity.** *Endocrine* 2005, **26:**1–9.
38. Miller TW, Shin I, Kagawa N, Evans DB, Waterman MR, Arteaga CL: **Aromatase is phosphorylated in situ at serine-118.** *J Steroid Biochem Mol Biol* 2008, **112:**95–101.
39. Barone I, Giordano C, Malivindi R, Lanzino M, Rizza P, Casaburi I, Bonofiglio D, Catalano S, Ando S: **Estrogens and PTP1B function in a novel pathway to regulate aromatase enzymatic activity in breast cancer cells.** *Endocrinology* 2012, **153:**5157–5166.
40. Christakos S, Dhawan P, Porta A, Mady LJ, Seth T: **Vitamin D and intestinal calcium absorption.** *Mol Cell Endocrinol* 2011, **347:**25–29.
41. Agoston A, Kunz L, Krieger A, Mayerhofer A: **Two types of calcium channels in human ovarian endocrine cells: involvement in steroidogenesis.** *J Clin Endocrinol Metab* 2004, **89:**4503–4512.
42. Paul S, Kundu S, Pramanick K, Bandyopadhyay A, Mukherjee D: **Regulation of ovarian steroidogenesis in vitro by gonadotropin in common carp Cyprinus carpio: interaction between calcium- and adenylate cyclase-dependent pathways and involvement of ERK signaling cascade.** *J Mol Endocrinol* 2010, **45:**207–218.
43. Weitzel JM, Vernunft A, Kruger B, Plinski C, Viergutz T: **LOX-1 regulates estrogenesis via intracellular calcium release from bovine granulosa cells.** *Cytometry A* 2014, **85:**88–93.
44. Reilly CM, Cannady WE, Mahesh VB, Stopper VS, De Sevilla LM, Mills TM: **Duration of estrogen exposure prior to follicle-stimulating hormone stimulation is critical to granulosa cell growth and differentiation in rats.** *Biol Reprod* 1996, **54:**1336–1342.
45. Emmen JM, Couse JF, Elmore SA, Yates MM, Kissling GE, Korach KS: **In vitro growth and ovulation of follicles from ovaries of estrogen receptor (ER) {alpha} and ER{beta} null mice indicate a role for ER{beta} in follicular maturation.** *Endocrinology* 2005, **146:**2817–2826.
46. Gougeon A: **Human ovarian follicular development: from activation of resting follicles to preovulatory maturation.** *Ann Endocrinol (Paris)* 2010, **71:**132–143.
47. Matsuda F, Inoue N, Manabe N, Ohkura S: **Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells.** *J Reprod Dev* 2012, **58:**44–50.
48. Kinuta K: **Regulation of vitamin D biosynthesis by calcium.** *Clin Calcium* 2003, **13:**899–903.
49. Wojtusik J, Johnson PA: **Vitamin D regulates anti-Mullerian hormone expression in granulosa cells of the hen.** *Biol Reprod* 2012, **86:**91.
50. Irani M, Merhi Z: **Role of vitamin D in ovarian physiology and its implication in reproduction: a systematic review.** *Fertil Steril* 2014, **102:**460–468. e463.

doi:10.1186/1477-7827-12-90
Cite this article as: Lee *et al.*: 1,25-Dihydroxyvitamin D3 increases testosterone-induced 17beta-estradiol secretion and reverses testosterone-reduced connexin 43 in rat granulosa cells. *Reproductive Biology and Endocrinology* 2014 **12**:90.

Cluster analysis of cardiovascular and metabolic risk factors in women of reproductive age

Chii-Ruey Tzeng, M.D.,[a] Yuan-chin Ivan Chang, Ph.D.,[b] Yu-chia Chang, M.Sc.,[b] Chia-Woei Wang, M.D.,[a] Chi-Huang Chen, M.D., Ph.D.,[a] and Ming-I Hsu, M.D.[c]

[a] Department of Obstetrics and Gynecology, Taipei Medical University Hospital, Taipei Medical University; [b] Institute of Statistical Science, Academia Sinica; and [c] Department of Obstetrics and Gynecology, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan

Objective: To study the association between endocrine disturbances and metabolic complications in women seeking gynecologic care.
Design: Retrospective study, cluster analysis.
Setting: Outpatient clinic, university medical center.
Patient(s): 573 women, including 384 at low risk and 189 at high risk of cardiometabolic disease.
Intervention(s): None.
Main Outcome Measure(s): Cardiovascular and metabolic parameters and clinical and biochemical characteristics.
Result(s): Risk factors for metabolic disease are associated with a low age of menarche, high levels of high-sensitivity C-reactive protein and liver enzymes, and low levels of sex hormone-binding globulin. Overweight/obese status, polycystic ovary syndrome, oligo/amenorrhea, and hyperandrogenism were found to increase the risk of cardiometabolic disease. However, hyperprolactinemia and premature ovarian failure were not associated with the risk of cardiometabolic disease. In terms of androgens, the serum total testosterone level and free androgen index but not androstenedione or dehydroepiandrosterone sulfate (DHEAS) were associated with cardiometabolic risk.
Conclusion(s): Although polycystic ovary syndrome is associated with metabolic risk, obesity was the major determinant of cardiometabolic disturbances in reproductive-aged women. Hyperprolactinemia and premature ovarian failure were not associated with the risk of cardiovascular and metabolic diseases.
Clinical Trial Registration Number: NCT01826357. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Cardiovascular risk, cluster analysis, metabolic syndrome, PCOS

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/tzengcr-cluster-analysis-cardiovascular-metabolic-risk-pcos/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

A cluster of risk factors for cardiovascular disease (CVD) and type 2 diabetes mellitus, which occur together more often than by chance, have become known as the metabolic syndrome (MetS) (1). The cardiovascular risk factors that comprise MetS have been recognized as a cluster since the 1920s (2). Although MetS and CVD are major causes of mortality for women of advanced age, the risks of MetS and CVD in reproductive-aged women are not well understood. Early detection of individuals at high risk for MetS by the use of accurate measures of insulin resistance (IR) could improve the detection and prevention of CVD and diabetes (3).

Received October 17, 2013; revised and accepted January 15, 2014.
C.-R.T. has nothing to disclose. Y.-c.I.C. has nothing to disclose. Y.-c.C. has nothing to disclose. C.-W.W. has nothing to disclose. C.-H.C. has nothing to disclose. M.-I.H. has nothing to disclose.
C.-R.T. and Y.-c.I.C. should be considered similar in author order.
Supported by the National Science Council [Grant NSC 102–2629-B-038–001] and Taipei Medical University, Wan Fang Hospital [Grant 101TMU-WFH-04].
Reprint requests: Ming-I Hsu, M.D., Department of Obstetrics and Gynecology, Wan Fang Hospital, Taipei Medical University, No. 111, Sec. 3, Xinglong Rd., Taipei 11696, Taiwan (E-mail: hsumingi@yahoo.com.tw).

Fertility and Sterility® Vol. ■, No. ■, ■ 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.01.023

Recent studies have suggested that there are some clinically relevant differences between women and men in terms of the prevalence, presentation, management, and outcomes of the disease, but little is known about why CVD affects women and men differently (4). Over recent decades, mortality rates in men have steadily declined while those in women have remained stable. This knowledge gap may explain why cardiovascular health in women is not improving as fast as that of men (4). In particular, the risk of developing MetS and CVD for younger women has not been well studied.

Although many aspects of CVD are similar in women and men, there is a growing body of evidence to support

sex and gender dimorphism in the prevalence, presenting symptoms, management, and outcomes of CVD (4). For instance, Lee et al. (5) reported that women with diabetes have a significantly higher CVD mortality rate than men with diabetes. Women of reproductive age present with cyclic endocrine changes that might result in different MetS and CVD risk factors in comparison with men. Menstrual cycle irregularity may be a marker of metabolic abnormalities predisposing women to an increased risk for CVD (6). The most well-known correlation between metabolic syndrome and reproductive disorders is in women with polycystic ovary syndrome (PCOS), which is diagnosed by hyperandrogenism and chronic anovulation. Although studies of PCOS and metabolic complications have been widely reported, the understanding of the correlation between endocrine status and metabolic complications in reproductive-aged women remains limited and controversial (7–10).

Definitions of metabolic syndrome are usually problematic because they are based on arbitrary cutoff points for several quantitative variables, where each variable is related linearly to cardiovascular risk (11). Further, the risks of developing MetS and CVD vary depending on race and gender. To understand the risk factors of cardiovascular and metabolic disease in reproductive-aged women, the studied subjects should be specified. Cluster analysis is a statistical method based on algorithms that aims to minimize within-group variation and maximize between-group variation for the clustering variables (11). This technique is suitable for defining groups and reflecting the natural structure of data without relying on inappropriate arbitrary cutoffs (12). Cluster analysis can be used to identify groups of women sharing similar metabolic risk factor patterns. We conducted this retrospective study on reproductive-aged Taiwanese women, and we used cluster analysis to investigate the relationship between metabolic complications and biochemical/clinical characteristics of endocrinologic dysfunction in women of reproductive age.

MATERIALS AND METHODS

This study was approved by the institutional review board of Taipei Medical University, Wan Fang Hospital, Taipei, Taiwan, with the identifier Hsu2013-TMU-JIRB 201302002 and registered at ClinicalTrials.gov with the identifier NCT01826357. We retrospectively reviewed the medical records of female patients who visited our reproductive endocrinology clinic from January 1, 2009, to June 31, 2012.

Parameters of Cardiovascular Risk

Metabolic syndrome is a complex of interrelated risk factors for CVD and diabetes. These factors include dysglycemia, high blood pressure, elevated triglyceride levels, low high-density lipoprotein (HDL) cholesterol levels, and central adiposity (1). To evaluate the risk of MetS and CVD, the following 10 cardiometabolic parameters were used for initial cluster analysis in this study: systolic blood pressure, diastolic blood pressure, waist size, fasting insulin, fasting glucose, 2-hour glucose, total cholesterol, triglyceride, HDL, and low-density lipoprotein (LDL).

Study Data

Women who had a complete set of anthropometric measurements and clinical and biochemical data about endocrinologic and cardiovascular parameters were initially included. For comparison with the healthy volunteers, the chief complaints of the studied patients were menstrual irregularity, infertility, overweight status, acne/hirsutism, and other conditions (i.e., more than one complaint, transfer from other medical specialists, headache, abdomen pain, vaginal itching, etc.) (Supplemental Table 1). The patients' medical histories included a detailed menstrual and medical/surgical history, anthropometric measurements (weight, height, waist, and hip), and blood pressure.

The dates and assays performed for blood sampling have been previously described elsewhere (13). The following data were collected and calculated: [1] serum androgens, including total testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEA-S), 17-α-OH progesterone, and free androgen index (FAI); [2] insulin sensitivity and glucose tolerance, including fasting insulin, fasting glucose, 2-hour glucose, and the homeostasis model assessment insulin resistance index (HOMA-IR); [3] lipid profiles, including total cholesterol, triglycerides, HDL, and LDL; [4] liver function and inflammatory markers, including glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and high sensitivity C-reactive protein (hs-CRP); and [5] sex hormone-binding globulin (SHBG), prolactin, and antimüllerian hormone (AMH).

The risks of MetS, impaired glucose tolerance, and diabetes were evaluated in every studied subject. The waist-to-hip ratio (WHR) was defined as waist circumference/hip circumference. Body mass index (BMI) was defined as the body weight in kilograms divided by the body height in meters squared (kg/m^2). Overweight/obese was defined as BMI $\geq$25 kg/m^2. All studied women received an ultrasonography examination. A vaginal ultrasound examination is preferred for young women without sexual experience, but we performed abdominal ultrasounds to detect polycystic ovaries.

Premature ovarian failure (POF) was defined as oligo/amenorrhea in women younger than 40 years with elevated serum FSH levels (FSH >16 mIU/mL). A diagnosis of POF was confirmed by serum examination of FSH 2 weeks later. Hyperprolactinemia was diagnosed when prolactin levels were above the upper limit of normal (24.20 ng/mL).

The following women were excluded from the study populations: [1] women who had been diagnosed with malignant tumors, Asherman syndrome, müllerian agenesis, or chromosomal anomalies; [2] women who had undergone menarche within the past 1 year or were older than 49 years; and [3] women who had received hormones and/or medicines for diabetes, hypertension, or dyslipidemia within the previous 3 months. A total of 713 women were included in the study for evaluation. To perform the cluster analysis, the above-mentioned 10 parameters were used to evaluate the risks of developing MetS and CVD (systolic pressure, diastolic pressure, waist size, fasting glucose, fasting insulin, 2-hour glucose, total cholesterol, triglyceride, HDL, and LDL).

To identify the clinical and biochemical characteristics in the high-risk MetS and CVD group, the studied patients were classified into two comparative groups. The classification of the high-risk and low-risk groups was performed by hierarchical cluster analysis using both within-group linkage and Ward's method. Using the within-group linkage method, 258 cases were classified as high-risk patients, and 455 were classified as low-risk patients. However, using Ward's analysis, 260 and 453 patients were classified as high risk and low risk, respectively. To verify the data, 189 and 384 patients who were confirmed to be in the high-risk and low-risk groups, respectively, with both methods were used for the final analysis, and 140 cases were further excluded due to inconsistent classification with the two different methods (Fig. 1). Women with missing endocrinologic or metabolic data were excluded. All included 573 cases featured complete data for all parameters.

We diagnosed PCOS according to the Androgen Excess and PCOS Society criteria (14), which require the presence of hyperandrogenism (hirsutism and/or biochemical) and ovarian dysfunction (oligoanovulation and/or polycystic ovaries). The definitions of oligoanovulation and polycystic ovaries have been described in detail elsewhere (15). Hyperandrogenism (HA) was defined as hirsutism and/or biochemical hyperandrogenemia. Biochemical hyperandrogenemia was defined as a total serum testosterone value $\geq$0.8 ng/mL (normal range for female adults is 0.1–0.8 ng/mL), androstenedione $\geq$2.99 ng/dL (normal range for females is 0.10–2.99 ng/mL), or DHEAS $\geq$275 μg/L (16). Hirsutism was defined as a modified Ferriman–Gallwey score of $\geq$6.

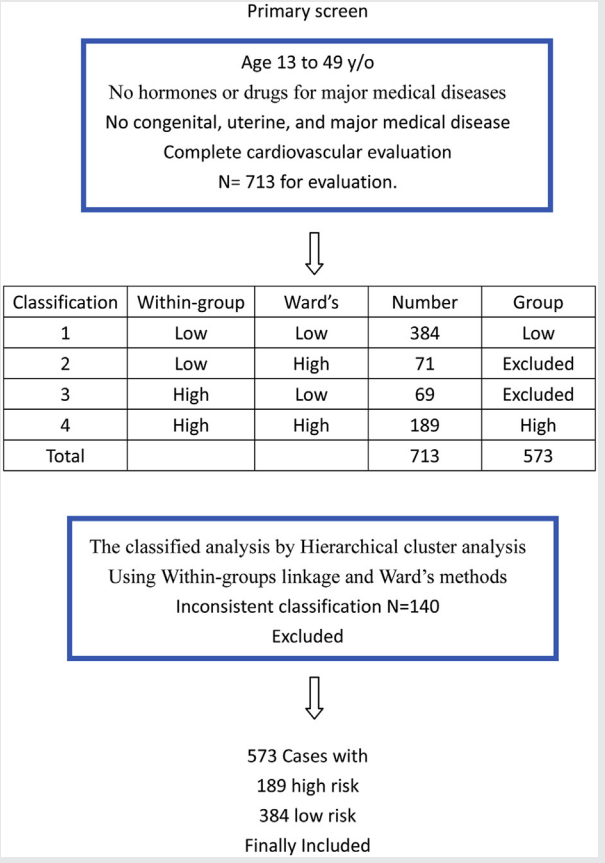
The insulin sensitivity index was evaluated by the homeostasis model assessment insulin resistance index (HOMA-IR) using the following formula:

$$\text{HOMA-IR} = \frac{\left[\text{Fasting insulin}\left(\frac{\mu U}{mL}\right) \times \text{Fasting glucose}\left(\frac{mg}{dL}\right)\right]}{405}$$

Impaired glucose tolerance (IGT) was defined as 2-hour glucose levels of 140–199 mg/dL in the 75-g oral glucose tolerance test. In women with IGT, the fasting plasma glucose (FPG) level should be less than 126 mg/dL.

Metabolic syndrome (MBS) was defined (2005 National Cholesterol Education Program Adult Treatment Panel III) as the presence of at least three of the following criteria: abdominal obesity (waist circumference >80 cm in women),

FIGURE 1



Flow chart of patient selection in this study.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

TABLE 1

Parameters of cardiovascular and metabolic risk in high-risk and low-risk women.

Parameter	Total	Low risk	High risk	*P* value
Case number	573	384	189	
Systolic pressure (mm Hg)	112.0 ± 17.1	104.9 ± 12.2	126.4 ± 16.4	<.001[a]
Diastolic pressure (mm Hg)	75.9 ± 13.0	70.7 ± 9.4	86.5 ± 13.0	<.001[a]
Waist (cm)	82.1 ± 15.3	73.4 ± 7.3	99.8 ± 11.6	<.001[a]
Fasting insulin (μIU/mL)	12.5 ± 12.1	8.0 ± 4.3	21.7 ± 16.8	<.001[a]
Fasting glucose (mg/dL)	90.2 ± 9.5	87.4 ± 6.6	95.8 ± 11.6	<.001[a]
2-hour glucose (mg/dL)	108.1 ± 32.6	96.3 ± 20.9	132.1 ± 38.4	<.001[a]
Cholesterol (mg/dL)	183.7 ± 32.1	185.0 ± 31.9	181.0 ± 32.3	.160
Triglycerides (mg/dL)	81.4 ± 68.7	60.6 ± 26.9	123.5 ± 101.0	<.001[a]
High-density lipoprotein (mg/dL)	56.2 ± 16.6	64.0 ± 14.3	40.4 ± 6.7	<.001[a]
Low-density lipoprotein (mg/dL)	107.1 ± 27.9	101.7 ± 26.7	118.0 ± 27.0	<.001[a]

Note: Data are mean ± standard deviation.
[a] *P*< .05.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

TABLE 2

Clinical and biochemical characteristics of women at high and low risk of cardiovascular and metabolic disorder.

Characteristic	Total	Low risk	High risk	*P* value
Case no.	573	384	189	
Age (y)	27.3 ± 6.5	26.9 ± 6.4	27.9 ± 6.6	.096
Menarche (y)	12.6 ± 1.5	12.7 ± 1.5	12.3 ± 1.4	.001[a]
hs-CRP[a] (mg/L)	0.22 ± 0.38	0.12 ± 0.27	0.44 ± 0.46	<.001[a]
SHBG (ng/dL)	45.8 ± 29.3	56.2 ± 29.1	24.9 ± 15.1	<.001[a]
Antimüllerian hormone (ng/mL)	7.46 ± 5.44	7.67 ± 5.47	7.02 ± 5.36	.175
Anthropometric measurements				
Weight (kg)	63.3 ± 17.4	53.4 ± 7.5	83.3 ± 14.4	<.001[a]
Height (cm)	160.5 ± 5.3	160.0 ± 5.1	161.3 ± 5.6	.008[a]
BMI (kg/m^2)	24.5 ± 6.5	20.8 ± 2.6	32.0 ± 5.3	<.001[a]
Hip (cm)	98.7 ± 11.6	92.6 ± 6.6	111.0 ± 9.7	<.001[a]
Waist to hip ratio	0.83 ± 0.09	0.79 ± 0.07	0.90 ± 0.07	<.001[a]
Hyperprolactinemia	10%	11%	7%	.121
POF	3%	4%	2%	.173
PCOS	47%	39%	61%	<.001[a]
Oligo/amenorrhea	56%	51%	64%	.003[a]
Polycystic ovary morphology	57%	54%	64%	.018[a]
Hyperandrogenism	54%	47%	67%	<.001[a]
Biochemical hyperandrogenemia	36%	30%	47%	<.001[a]
Hirsutism	34%	29%	43%	.001[a]
Metabolism				
Metabolic syndrome	22%	1%	66%	<.001[a]
Hypertension	27%	10%	62%	<.001[a]
HDL <50 mg/dL	39%	14%	92%	<.001[a]
Triglycerides >150 mg/dL	10%	2%	26%	<.001[a]
Waist >80 cm	45%	18%	98%	<.001[a]
Impaired glucose tolerance	9%	2%	22%	<.001[a]
Diabetes mellitus	2%	0	7%	<.001[a]
Androgens				
Total testosterone (ng/mL)	0.60 ± 0.29	0.54 ± 0.26	0.72 ± 0.33	<.001[a]
Androstenedione (ng/dL)	2.71 ± 1.37	2.66 ± 1.36	2.79 ± 1.40	.301
Free androgen index	7.68 ± 8.30	4.59 ± 3.78	13.9 ± 11.0	<.001[a]
DHEAS (ng/dL)	197.3 ± 103.4	193.9 ± 97.9	204.1 ± 113.7	.271
17-OH PRG (ng/dL)	1.14 ± 0.99	1.20 ± 1.11	1.02 ± 0.69	.041[a]
mFG score	5.80 ± 4.73	5.27 ± 4.18	6.90 ± 5.54	<.001[a]
Insulin sensitivity and liver function				
HOMA-IR	2.92 ± 3.25	1.75 ± 0.97	5.29 ± 4.67	<.001[a]
GOT IU/L	23.4 ± 11.6	20.6 ± 6.5	29.0 ± 06.6	<.001[a]
GPT IU/L	23.1 ± 19.7	17.0 ± 9.2	35.6 ± 27.8	<.001[a]

Note: Data are either mean ± SD or are percentage. BMI = body mass index; DHEA-S = dehydroepiandrosterone sulfate; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; HDL = high-density lipoprotein; HOMA-IR = The homeostasis model assessment insulin resistance index; hs-CRP = high-sensitivity C-reactive protein; modified mFG Score = Ferriman–Gallwey score; 17-OH PRG = 17-α-OH progesterone; PCOS = polycystic ovary syndrome; POF = premature ovarian failure; SHBG = sex hormone-binding globulin.

[a] $P<.05$.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

serum triglycerides ≥150 mg/dL, serum HDL <50 mg/dL, systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg, and fasting plasma glucose ≥100 mg/dL.

Statistical Analysis

We performed cluster analysis to identify groups of women with similar cardiometabolic risk factor patterns using two-step cluster analysis with Statistical Package for Social Science 15.0 software (SPSS Institute). The number of clusters is determined automatically.

In Tables 1 and 2, data are presented as the mean ± standard deviation. We used chi-square and Fisher's exact tests to perform categorical comparisons and analysis of variance (ANOVA) to compare the continuous variables. The means of more than two groups were compared using one-way ANOVA post hoc range (Dunnett's) tests with equal variances not assumed. For differences between the groups, $P<.05$ was considered statistically significant.

RESULTS

Among the 573 studied patients, 384 and 189 patients were classified as being low and high risk, respectively. For clinical diagnosis, 235 (47%) subjects had PCOS, 55 (10%) had hyperprolactinemia, and 17 (3%) had POF. In terms of metabolic complications, 128 (22%) had MetS, 48 (9%) had impaired glucose tolerance, and 13 (2%) had diabetes mellitus.

Table 1 shows the 10 parameters related to cardiovascular and metabolic risk in the high- and low-risk groups. Nine out of 10 parameters (all except serum total cholesterol level) were statistically significantly different between the two groups ($P<.001$). The results show that the above classifications can be used to separate high- and low-risk patients in terms of cardiovascular and metabolic risk.

Table 2 presents the clinical and biochemical characteristics of patients at high and low risk of cardiovascular and metabolic diseases. All parameters related to metabolic risk and insulin resistance were statistically significantly different between the high- and low-risk groups. Patients with hyperprolactinemia and POF did not exhibit an increased cardiovascular and metabolic risk. In contrast, the high-risk group had higher prevalences of hyperandrogenism, oligomenorrhea, polycystic ovary morphology, and PCOS. In terms of androgens, the high-risk group had a higher serum total testosterone and free androgen index than the low-risk group; however, the serum androstenedione and DHEAS levels did not differ between the high- and low-risk groups.

Figure 2 shows the odds ratios (OR) for patients at high risk for cardiovascular and metabolic disease. Overweight/obese (OR 11.2, 95% confidence interval [CI]: 8.0–15.7), PCOS (OR 1.6; 95% CI, 1.3–1.9), oligo/amenorrhea (OR 1.3; 95% CI, 1.1–1.4), polycystic ovary morphology (OR 1.2; 95% CI, 1.0–1.4), hyperandrogenism (OR 1.4; 95% CI, 1.2–1.6), and biochemical hyperandrogenemia (OR 1.6; 95% CI, 1.3–1.9). Hyperprolactinemia (OR 0.6; 95% CI, 0.3–1.1) and POF (OR 0.4; 95% CI, 0.1–1.5) were not associated with an increased risk of cardiovascular and metabolic diseases.

DISCUSSION

The metabolic syndrome is a cluster of cardiometabolic factors that predisposes individuals to diabetes and CVD. The question of whether MetS is a simple aggregation of associated metabolic risk factors or a true syndrome arising from a common physiologic origin is controversial (17). Even congruent definitions differ between international authorities and continue to change (18). Cutoff values for risk factors should be determined, and these definitions should reflect gender-based and ethnic differences. To avoid the bias of gender and ethnic variance, our study included reproductive-aged Taiwanese women only.

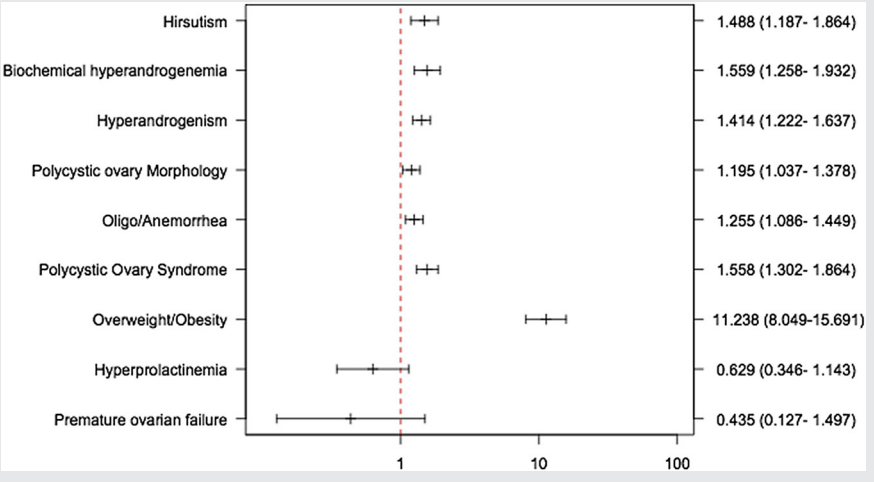
Instead of using arbitrary cutoff values, we performed cluster analysis using 10 cardiovascular and metabolic risk parameters and classified the study population into high- and low-risk groups. Cluster analysis was a useful tool for identifying groups of women sharing similar metabolic risk factor patterns.

The main purpose of this study was to identify the clinical and biochemical characteristics of high and low metabolic risk in reproductive women. To confirm the classification of the high- and low-risk groups, two cluster analysis methods were applied and confirmed in our study. We excluded 140 patients who could not be consistently classified by the two statistical methods. Finally, the parameters related to cardiovascular disease, metabolic syndrome, and insulin resistance were significantly different between the high- and low-risk groups. The metabolic syndrome was diagnosed by the cluster of abdominal obesity, hyperglycemia, hypertension, and dyslipidemia, which increases the risk for type 2 diabetes and cardiovascular diseases (19). We believe this classification could separate the high and low MetS and CVD risk groups in our studied populations.

The metabolic profile noted in women with PCOS is similar to that of insulin resistance syndrome and consists of hyperinsulinemia, mild glucose intolerance, dyslipidemia, and hypertension (20). Nine out of 10 parameters, all except total cholesterol, that are related to MetS and CVD risk (blood pressure, waist size, insulin, glucose, triglyceride, HDL, and LDL) were strongly associated with the high-risk group in our study. The association between PCOS and metabolic disturbance has been widely reported.

The risk of MetS may vary among the four phenotypes of PCOS based on the Rotterdam criteria (9). Hyperinsulinemia was reported to correlate with free testosterone levels only in women with traditional U.S. National Institutes of Health–defined PCOS (7). Goverde et al. (21) reported that hyperandrogenic PCOS phenotypes are strongly linked to MetS and insulin resistance in Dutch women with PCOS. Legro et al.

FIGURE 2



Odds ratio for the high risk of a clinical diagnosis of cardiovascular disease.

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

(8) reported that neither the morphology nor the volume of the ovaries is associated with distinctive metabolic or reproductive phenotypes in women with PCOS. In most patients, PCOS-related disturbances (hyperandrogenism, ovulatory dysfunction, and polycystic ovary morphology) may cluster together, making it difficult to evaluate them individually. Our study shows that all PCOS-related disorders, such as ovulatory dysfunction, hyperandrogenism, hirsutism, and polycystic ovary morphology, clustered together and were strongly associated with high risk of MetS and CVD. Furthermore, the high-risk group had higher levels of inflammatory markers and liver enzymes and lower SHBG levels than the low-risk group. It is interesting that the high-risk group had an earlier menarche than the low-risk group. We found that hyperprolactinemia and POF were not related with MetS and CVD risk. Androgen levels are the major distinguishing endocrine feature among the phenotypic expressions of PCOS. Androgen excess in women may signal a risk for coronary artery disease (20). However, the different types of androgens should be evaluated separately. Specifically, the serum total testosterone level and free androgen index, but not androstenedione or DHEAS, were associated with the risk of MetS and CVD.

Obesity should be the major impact factor for reproductive-aged women with a high risk of MetS and CVD. The association between obesity and a cluster of cardiometabolic risk factors is stronger in women than in men, and this gender-specific difference exists in younger but not in older individuals (22). Obesity accounts for the maximum variance in clustering and appears to be a more powerful correlate of cardiovascular risk in children and adolescents (23). Ketel et al. (24), using a isoglycemic-hyperinsulinemic clamp, showed that PCOS per se is not associated with impaired metabolic insulin sensitivity in normal-weight women, but it aggravates the impairment of metabolic insulin sensitivity in obese women. Spranger et al. (25) measured the insulin resistance of women with PCOS by the continuous infusion of glucose and model assessment and demonstrated that BMI but not testosterone was independently associated with insulin sensitivity. Similarly, in our study, obesity was the major factor determining the risks of MetS and CVD in reproductive-aged women. Although PCOS and obesity both increased the risks of MetS and CVD, the OR in high-risk women with PCOS (OR 1.6; 95% CI, 1.3–1.9) was much lower than that for women who were overweight/obese (OR 11.2; 95% CI, 8.0–15.7).

The major weakness of our study is that the women we evaluated were recruited from the outpatient clinic of a tertiary care center and thus do not reflect the true distribution of the general population. Therefore, the results should be applied to the general population with caution.

We can summarize our studied results with the following points. [1] Overweight/obese status was the major determinant of cardiovascular and metabolic disturbances in reproductive-aged women. [2] Early menarche, high levels of inflammatory markers and liver enzymes, and low SHBG were associated with high cardiovascular and metabolic risk. [3] Hyperprolactinemia and POF were not associated with cardiovascular and metabolic risk. [4] Oligomenorrhea, hyperandrogenism, and PCOS were associated with high cardiovascular and metabolic risk. [5] The serum total testosterone level and free androgen index, but not androstenedione or DHEAS, were associated with cardiovascular and metabolic risk.

REFERENCES

1. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 2009;120:1640–5.
2. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. Lancet 2005; 365:1415–28.
3. Taverna MJ, Martínez-Larrad MT, Frechtel GD, Serrano-Ríos M. Lipid accumulation product: a powerful marker of metabolic syndrome in healthy population. Eur J Endocrinol 2011;164:559–67.
4. Pilote L, Dasgupta K, Guru V, Humphries KH, McGrath J, Norris C, et al. A comprehensive view of sex-specific issues related to cardiovascular disease. CMAJ 2007;176(Suppl):S1–44.
5. Lee WL, Cheung AM, Cape D, Zinman B. Impact of diabetes on coronary artery disease in women and men: a meta-analysis of prospective studies. Diabetes Care 2000;23:962–8.
6. Solomon CG, Hu FB, Dunaif A, Rich-Edwards JE, Stampfer MJ, Willett WC, et al. Menstrual cycle irregularity and risk for future cardiovascular disease. J Clin Endocrinol Metab 2002;87:2013–7.
7. Kauffman RP, Baker TE, Baker VM, DiMarino P, Castracane VD. Endocrine and metabolic differences among phenotypic expressions of polycystic ovary syndrome according to the 2003 Rotterdam consensus criteria. Am J Obstet Gynecol 2008;98:670.e1–7.
8. Legro RS, Chiu P, Kunselman AR, Bentley CM, Dodson WC, Dunaif A. Polycystic ovaries are common in women with hyperandrogenic chronic anovulation but do not predict metabolic or reproductive phenotype. J Clin Endocrinol Metab 2005;90:2571–9.
9. Shroff R, Syrop CH, Davis W, Van Voorhis BJ, Dokras A. Risk of metabolic complications in the new PCOS phenotypes based on the Rotterdam criteria. Fertil Steril 2007;88:1389–95.
10. Welt CK, Gudmundsson JA, Arason G, Adams J, Palsdottir H, Gudlaugsdottir G, et al. Characterizing discrete subsets of polycystic ovary syndrome as defined by the Rotterdam criteria: the impact of weight on phenotype and metabolic features. J Clin Endocrinol Metab 2006;91: 4842–8.
11. Huang RC, Burke V, Newnham JP, Stanley FJ, Kendall GE, Landau LI, et al. Perinatal and childhood origins of cardiovascular disease. Int J Obes (Lond) 2007;31:236–44.
12. Hart R, Doherty DA, Mori T, Huang RC, Norman RJ, Franks S, et al. Extent of metabolic risk in adolescent girls with features of polycystic ovary syndrome. Fertil Steril 2011;95:2347–53, 2353.e1.
13. Liang SJ, Hsu CS, Tzeng CR, Chen CH, Hsu MI. Clinical and biochemical presentation of polycystic ovary syndrome in women between the ages of 20 and 40. Hum Reprod 2011;26:3443–9.
14. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. Task Force on the Phenotype of the Polycystic Ovary Syndrome of the Androgen Excess and PCOS Society. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. Fertil Steril 2009;91:456–88.
15. Hsu MI, Liou TH, Liang SJ, Su HW, Wu CH, Hsu CS. Inappropriate gonadotropin secretion in polycystic ovary syndrome. Fertil Steril 2009;91:1168–74.
16. Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab 2004;89:2745–9.
17. Yoon JH, Park JK, Oh SS, Lee KH, Kim SK, Kim JK, et al. The clustering patterns of metabolic risk factors and its association with sub-clinical atherosclerosis in Korean population. Ann Hum Biol 2011;38:640–6.

18. Jørgensen ME, Bjerregaard P, Gyntelberg F, Borch-Johnsen K; Greenland Population Study. Prevalence of the metabolic syndrome among the Inuit in Greenland: a comparison between two proposed definitions. Diabet Med 2004;21:1237–42.
19. Povel CM, Beulens JW, van der Schouw YT, Dollé ME, Spijkerman AM, Verschuren WM, et al. Metabolic syndrome model definitions predicting type 2 diabetes and cardiovascular disease. Diabetes Care 2013;36:362–8.
20. Wild RA, Grubb B, Hartz A, Van Nort JJ, Bachman W, Bartholomew M. Clinical signs of androgen excess as risk factors for coronary artery disease. Fertil Steril 1990;54:255–9.
21. Goverde AJ, van Koert AJ, Eijkemans MJ, Knauff EA, Westerveld HE, Fauser BC, et al. Indicators for metabolic disturbances in anovulatory women with polycystic ovary syndrome diagnosed according to the Rotterdam consensus criteria. Hum Reprod 2009;24:710–7.
22. Wakabayashi I. Age-dependent influence of gender on the association between obesity and a cluster of cardiometabolic risk factors. Gend Med 2012;9:267–77.
23. Khader YS, Batieha A, Jaddou H, Batieha Z, El-Khateeb M, Ajlouni K. Factor analysis of cardiometabolic risk factors clustering in children and adolescents. Metab Syndr Relat Disord 2011;9:151–6.
24. Ketel IJ, Stehouwer CD, Serné EH, Korsen TJ, Hompes PG, Smulders YM, et al. Obese but not normal-weight women with polycystic ovary syndrome are characterized by metabolic and microvascular insulin resistance. J Clin Endocrinol Metab 2008;93:3365–72.
25. Spranger J, Möhlig M, Wegewitz U, Ristow M, Pfeiffer AF, Schill T, et al. Adiponectin is independently associated with insulin sensitivity in women with polycystic ovary syndrome. Clin Endocrinol (Oxf) 2004;61:738–46.

SUPPLEMENTAL TABLE 1

Chief complaints of 573 studied patients.

Chief complaint	Case no.	Age (y)	Percentage
Menstrual irregularity	274	26.3 ± 5.8	47.8
Healthy volunteer	60	26.3 ± 5.9	10.5
Infertility	49	33.6 ± 3.7	8.6
Overweight/obese	44	25.3 ± 6.4	7.7
PCOS transferred	43	26.4 ± 7.1	7.5
Acne/hirsutism	29	26.9 ± 7.2	5.1
Abnormal vaginal bleeding	10	25.3 ± 9.2	1.7
Other[a]	64	29.9 ± 6.8	11.2
Total	573	27.3 ± 6.5	100

Note: Data are mean ± standard deviation. PCOS = polycystic ovary syndrome.
[a] Patients who presented with more than one complaint or with other complaints (transferred from other medical specialists, headache, abdominal pain, vaginal itching, etc.).

Tzeng. Cardiometabolic risk in younger women. Fertil Steril 2014.

科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/16

科技部補助計畫	計畫名稱: 台灣女性血清中發炎標識、氧化壓標識及環境荷爾蒙標識對月經週期及女生殖荷爾蒙的影響
	計畫主持人: 徐明義
	計畫編號: 102-2629-B-038-001- 學門領域: 性別主流科技計畫

無研發成果推廣資料

102 年度專題研究計畫研究成果彙整表

計畫主持人：徐明義	計畫編號：102-2629-B-038-001-
計畫名稱：台灣女性血清中發炎標識、氧化壓標識及環境荷爾蒙標識對月經週期及女生殖荷爾蒙的影響	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	3	3	100%	篇	1. Tzeng CR, Chang YC, Chang YC, Wang CW, Chen CH, Hsu MI(2014). Cluster analysis of cardiovascular and metabolic risk factors in women of reproductive age. Fertil Steril.(101):1404-1410. 2.Lee CT, Wang JY, Chou KY, Hsu MI(2014). 1,25-dihydroxyvitamin D3 increases testosterone-induced 17beta-estradiol secretion and reverses testosterone-reducedconnexin 43 in rat granulosa cells. Reprod Biol Endocrinol.():-. 3.Ko PC, Huang SY, Hsieh CH, Hsu MI, Hsu CS(2014). Serum Ferritin levels and polycystic ovary syndrome in obese and non-obese women. Taiwan J Obstet Gynecol.():-.
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	

		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果(無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)		無					

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

本研究利用集群方法分析數據資料，將納入的 573 位女性分成具低風險(384 人)和高風險(159 人)心血管疾病兩組，探討心血管疾病和代謝症候群之間的關係。研究結果發現過重或肥胖、寡經或無經症以及高雄性激素症會提高心血管疾病和代謝疾病之風險，而目前的研究中已知女性常見的多囊性卵巢症候群與代謝症候群相關，但肥胖是生育年齡婦女造成心血管疾病和代謝症候群最主要的原因。因此，我們希望藉由女性內分泌檢查和代謝相關指標來尋找對女性適當的醫療照護。