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Mitochondrial DNA copy number in peripheral blood in polycystic ovary syndrome

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ABSTRACT

Polycystic ovary syndrome (PCOS) is associated with insulin resistance and various metabolic diseases; and recently, elevated oxidative stress has been detected in PCOS. Mitochondria are highly susceptible to oxidative damage; and disordered mitochondrial function at the cellular level can impact whole-body metabolic homeostasis, leading to the hypothesis that abnormalities in markers of mitochondrial metabolism are related to PCOS. We compared mitochondrial DNA (mtDNA) copy number in women with and without PCOS and investigated the independent relationship between mtDNA copy number and PCOS after adjustment for metabolic parameters. Fifty women with PCOS and 60 age- and body mass index–matched healthy women were studied. Mitochondrial DNA copy numbers as well as metabolic parameters and indices of insulin resistance were assessed. Mitochondrial DNA copy numbers were significantly lower in women with PCOS ($P < .01$). In the PCOS group, mtDNA copy number was negatively correlated with indices of insulin resistance, waist circumference, and triglyceride levels and positively correlated with sex hormone–binding globulin levels. In multiple logistic regression, the corresponding odds ratios (95% confidence interval) for PCOS by log-transformed mtDNA copy number and homeostasis model assessment of insulin resistance were 0.15 (0.04-0.56) and 4.26 (1.43-12.68), respectively, after adjustment for age, body mass index, and other metabolic factors. We report decreased mtDNA copy numbers in PCOS patients in relation to controls independently of insulin resistance or other metabolic factors. The pathophysiological and clinical significance of this finding requires further investigation.

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1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders of reproductive-age women and is characterized by hirsutism, chronic anovulation, and infertility [1]. Polycystic ovary syndrome is also associated with insulin resistance and various metabolic diseases [2,3]. However, the etiology of PCOS is complex and multifactorial; and its pathogenesis is not fully understood.

Mitochondria play a central role in energy production and are a major source of reactive oxygen species (ROS) [4]. This renders them highly susceptible to oxidative damage, and disordered mitochondrial function at the cellular level can impact whole-body metabolic homeostasis [5]. Recently, the concept that mitochondrial dysfunction might be a factor leading to insulin resistance and type 2 diabetes mellitus has been gaining wide acceptance, although there are still several unresolved issues [6].

In PCOS, elevated oxidative stress has been involved in the pathophysiology of anovulation [7]; and this stress was associated with hyperglycemia and hyperandrogenism [8]. In addition, Skov et al [9] have recently demonstrated reduced expression of genes involved in mitochondrial oxidative phosphorylation in skeletal muscle of insulin-resistant women with PCOS. These emerging data support a hypothesis that abnormalities in markers of mitochondrial metabolism are related to PCOS.

The mitochondrial DNA (mtDNA) copy number is a relative measure of the cellular number or mass of mitochondria. Mitochondrial DNA damage is considered to be an alternate readout of mitochondrial dysfunction because it leads to reduced cellular metabolic activity [10]. To our knowledge, there have not been any studies addressing the role of mtDNA content in PCOS.

The aim of our study was to compare mtDNA copy number in women with and without PCOS, with similar body mass index (BMI) and age, and to investigate the independent relationship between mtDNA copy number and PCOS after adjustment for metabolic parameters including insulin resistance.

2. Methods

2.1. Subjects

We recruited 50 women who had been diagnosed with PCOS based on the revised diagnostic criteria announced in the 2003 Rotterdam consensus [1]. Polycystic ovary syndrome was diagnosed when the phenotypes of the patients satisfied 2 of the following 3 criteria: oligomenorrhea or amenorrhea, clinical or biochemical hyperandrogenism, and ultrasonographic polycystic ovarian morphology. Patients with Cushing syndrome, late-onset congenital adrenal hyperplasia, thyroid dysfunction, hyperprolactinemia, and androgen-secreting tumors were excluded. Other exclusion criteria included diabetes, hypertension, chronic renal disease, smoking, and the use of alcohol or medications.

Sixty healthy, age- and BMI-matched women served as controls. The control women were not hirsute and had normal androgen levels. They all had normal, regularly cycling menstrual periods; normal appearance of their ovaries on ultrasound; and normal luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels. None of the women in the study were taking oral contraceptives. All participants gave their informed consent. The study was approved by the Institutional Review Board of Severance Hospital.

2.2. Assessment of clinical parameters

Body mass index was calculated as weight (kilograms) divided by the square of the height (square meters). Waist circumference was measured at the umbilicus while the subject was standing. Measurements were conducted within the first 10 days from the onset of menstruation in the normal controls and in cases of PCOS with mild oligomenorrhea. Measurements were conducted at random times for women who had severe oligomenorrhea or amenorrhea. Blood samples were collected in the morning between 7:00 AM and 10:00 AM after an overnight fast. Luteinizing hormone, FSH, estradiol (E_2), progesterone, total testosterone, and fasting insulin levels were measured by electrochemiluminescence immunoassays (Roche, Indianapolis, IN). Sex hormone–binding globulin (SHBG) levels were assayed using an Immulite 2000 analyser (DPC, Los Angeles, CA). Serum prolactin and thyroid hormone levels were verified as within the normal limits in all patients.

Fasting glucose, total cholesterol, triglyceride (TG), and high-density lipoprotein (HDL) cholesterol were measured by an ADVIA 1650 chemistry system (Bayer, Tarrytown, NY). Low-density lipoprotein (LDL) cholesterol levels were calculated using the Friedewald equation if the serum TG level was less than 400 mg/dL. High-sensitivity C-reactive protein (hs-CRP) was measured by a latex-enhanced immunoturbidimetric assay using an ADVIA 1650 Chemistry system (Bayer). *Clinical hyperandrogenism* was defined as the presence of acne, hirsutism (Ferriman-Gallwey score >7), or alopecia. Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) index, whereas insulin sensitivity was estimated by the quantitative insulin sensitivity check index (QUICKI). The calculations used for these estimations were as follows: HOMA-IR = [fasting insulin (micro–international units per milliliter) × fasting glucose (millimoles per liter)/22.5]; QUICKI = 1/{log [fasting insulin (micro–international units per milliliter)] + log [fasting glucose (milligrams per deciliter)]}.

2.3. Quantification of mtDNA copy number in peripheral blood

Peripheral blood mtDNA was extracted from 1 mL of whole blood with the QIAamp Tissue Kit 250 (Qiagen, Valencia, CA) according to the manufacturer's instructions. The relative mtDNA copy number was measured by a real-time polymerase chain reaction (QPCR) and corrected by simultaneous measurement of the nuclear DNA according to the method developed by Wong and Cortopassi [11] with some modifications [4]. Essentially, measurements were carried out using the Light Cycler-Fast Start DNA Master SYBR Green I kit supplied by Roche Molecular Biochemicals (Pleasanton, CA). Two primers (forward: 5′-GAAGAGCCAAGGACAGGTAC-3′; reverse: 5′-CAACTTCATCCACGTTCACC-3′) complementary

to the sequences of the β-globin gene were used to amplify a 268–base pair product. Real-time polymerase chain reaction was performed under the following conditions: initial denaturation at 95°C for 300 seconds followed by 40 cycles of 0.1 second at 95°C, 6 seconds at 58°C, and 18 seconds at 72°C. To identify the mitochondrial gene products, the forward primer 5′-AACATACCCATGGCCAACCT-3′ and the reverse primer 5′-AGCGAAGGGTTGTAGTAGCCC-3′, which are complementary to the sequence of the mitochondrial ND1 gene, were used to amplify a 153–base pair PCR product. Real-time polymerase chain reaction was performed under identical conditions except that the extension time was 8 seconds. A total of 20 ng of DNA was used in QPCR for the determination of the threshold cycle number (Ct) of the nuclear and mitochondrial genes, respectively. The Ct values for the nuclear β-globin gene and mitochondrial ND1 gene were determined simultaneously in each QPCR run. The intraassay and interassay coefficients of variation of Ct values for the ND1 gene were 4.5% and 5.8%, respectively. The Ct values can be used as a measure of the input copy number, and Ct value differences were used to quantify mtDNA copy number relative to the β-globin gene according to the following equation: relative copy number = 2ΔCt [12], where ΔCt is the Ctβ-globin – CtND1. Although we did not double-check all the samples, preliminary laboratory values correlated significantly when repeating the measurement using partial individuals ($r = 0.758$, $P < .01$). To reduce variations in measurements, all parameters throughout the study were measured by the same person.

2.4. *Statistical analysis*

All data were analyzed using the SAS 9·1 (SAS Institute, Cary, NC) statistical program, and $P < .05$ was considered statistically significant. Data are described as the mean ± SD for normal distributions and as median and interquartile range for nonnormal distributions. Measurements with a skewed distribution were normalized by logarithmic transformation. The Student *t* test or the nonparametric Wilcoxon rank sum test was used to test for demographic and laboratory differences between women with PCOS and controls. Pearson correlation coefficients were calculated to evaluate the relationship between log-transformed mtDNA copy number and metabolic risk factors in both the PCOS and control groups. In addition, the associations of log-transformed mtDNA copy numbers and metabolic factors with PCOS were measured by logistic regression analyses.

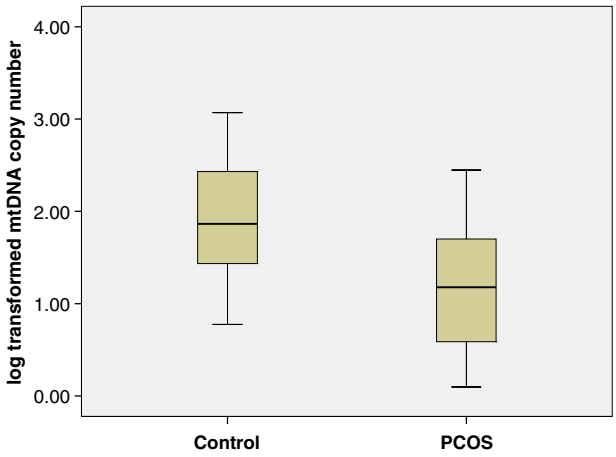

Fig. 1 – Log-transformed mtDNA copy number in women with PCOS and controls. *P* values (<.01) calculated by the *t* test.

3. Results

The clinical, hormonal, and biochemical features of PCOS patients and control subjects are summarized in Table 1.

Table 1 – Clinical characteristics of women with PCOS and control subjects

Characteristics	PCOS (n = 50)	Control (n = 60)	*P* value
Age (y)	29.1 ± 4.1	28.9 ± 4.5	.77
BMI (kg/m^2)	24.7 ± 4.4	25.4 ± 3.5	.40
Waist circumference(cm)	86.0 ± 12.2	87.1 ± 9.2	.84
Systolic BP (mm Hg)	116.4 ± 12.4	120.1 ± 11.9	.11
Diastolic BP (mm Hg)	75.6 ± 10.1	73.1 ± 8.2	.21
Fasting glucose (mmol/L)	5.23 ± 0.47	4.98 ± 0.45	.01
Fasting insulin (pmol/L)	62.5 (26.4-113.2)	40.0 (28.6-53.0)	<.01
HOMA-IR	2.1 (0.8-3.7)	1.3 (0.9-1.8)	<.01
QUICKI	0.15 ± 0.02	0.16 ± 0.01	.01
Total cholesterol (mmol/L)	4.53 ± 0.83	4.58 ± 0.69	.65
TG (mmol/L)	1.12 (0.72-1.52)	0.87 (0.62-1.02)	<.01
HDL cholesterol (mmol/L)	1.41 ± 0.35	1.49 ± 0.28	.18
LDL cholesterol (mmol/L)	2.51 ± 0.92	2.68 ± 0.60	.28
hs-CRP (μg/L)	15.7 (7.7-53.0)	30.0 (9.3-56.0)	.48
LH/FSH (IU/L)	1.25 (0.93-2.22)	1.24 (0.93-2.22)	.66
E_2 (pmol/L)	118.4 (87.7-162.7)	175.3 (101.9-496.4)	.53
Testosterone (nmol/L)	1.46 (1.01-2.71)	1.32 (1.01-1.87)	.46
SHBG (nmol/L)	40.4 (22.8-66.2)	53.0 (38.2-64.9)	.03

Data shown as mean ± SD or median with interquartile range (25th-75th percentile). *P* value are calculated by the *t* test (normally distributed variables) or Wilcoxon rank sum test (nonnormally distributed variables). BP indicates blood pressure.

Table 2 – Correlation of log-transformed mtDNA copy number and metabolic factors in women with PCOS and in control subjects

	Log-transformed mtDNA copy number			
	PCOS		Control	
	R	P value	R	P value
BMI (kg/m^2)	−0.29	.05	−0.11	.43
Waist circumference (cm)	−0.51	<.01	−0.06	.67
Systolic BP (mm Hg)	−0.08	.59	0.06	.67
Diastolic BP (mm Hg)	−0.15	.30	0.11	.46
Fasting glucose (mmol/L)	−0.05	.75	0.07	.61
Fasting insulin (pmol/L)	−0.38	<.01	−0.31	.02
HOMA-IR	−0.37	<.01	−0.28	.04
QUICKI	0.34	.02	0.32	.02
Total cholesterol (mmol/L)	−0.03	.86	0.02	.90
LDL cholesterol (mmol/L)	0.11	.45	−0.02	.91
HDL cholesterol (mmol/L)	0.12	.42	0.10	.44
TG (mmol/L)	−0.37	<.01	−0.04	.78
hs-CRP(μg/L)	−0.11	.45	0.04	.76
LH (IU/L)	0.22	.12	−0.27	.06
FSH (IU/L)	0.13	.43	−0.27	.11
E_2 (pmol/L)	−0.02	.88	−0.01	.96
Testosterone (nmol/L)	−0.16	.26	−0.35	.01
SHBG (nmol/L)	0.45	<.01	0.03	.85

Measurements with a skewed distribution were normalized by logarithmic transformation. Coefficients (r) and P values were calculated using the Pearson correlation model.

Compared with the healthy controls, women with PCOS had significantly higher fasting glucose, insulin, HOMA-IR, and TG levels, as well as significantly lower QUICKI and SHBG levels.

The mtDNA copy numbers were significantly lower in the patients with PCOS ($P < .01$). Logarithmic transformation was used because the original values of the relative mtDNA copy number were not normally distributed (Fig. 1).

In both PCOS patients and control subjects, mtDNA copy numbers were negatively correlated with fasting insulin and HOMA-IR and were positively correlated with QUICKI values. In PCOS patients, we found a negative correlation between mtDNA copy number and both waist circumference and TG levels, and a positive correlation of mtDNA copy number with SHBG levels. In control subjects, we found a negative correlation between mtDNA copy number and testosterone levels (Table 2).

Table 3 shows the risk for PCOS according to mtDNA copy numbers and metabolic factors. In multiple logistic regression, the corresponding odds ratios (95% confidence interval [CI]) for PCOS by log-transformed mtDNA copy number and HOMA-IR were 0.15 (0.04-0.56) and 4.26 (1.43-12.68), respectively, after adjustment for age and BMI and other metabolic factors. This implies that the association between PCOS and mtDNA copy number is independent from insulin resistance or other metabolic parameters.

4. Discussion

Besides suffering from reproductive abnormalities, insulin resistance, dyslipidemia, type 2 diabetes mellitus, and cardiovascular disease have recently come into focus in PCOS patients [13,14]. However, the mechanisms underlying metabolic disturbance in PCOS patients are poorly understood.

Increased mitochondrial ROS generation and defects have been implicated in the pathogenesis of insulin resistance and cardiovascular disease [15-17]. Recent experimental studies suggest that alterations in mtDNA play a fundamental role in the increase in ROS [18,19] and that the maintenance of mtDNA copy number is essential for the preservation of mitochondrial function and cell growth [20].

In our study, we found that mtDNA copy numbers in peripheral blood were significantly lower in women with PCOS compared with age- and BMI-matched healthy women and that mtDNA copy number had independent associations with PCOS. In addition, mtDNA copy numbers were negatively correlated with insulin resistance indices and TG levels and were positively correlated with SHBG. Reduced SHBG not only provides increased androgen bioactivity [21], but also is closely associated with insulin resistance and metabolic syndrome [22]. These findings suggest that mtDNA depletion may be involved in the pathogenesis of PCOS and may be part of one of the putative links between PCOS and metabolic and hormonal complications.

The mechanistic reason for decreased peripheral mtDNA copy numbers in PCOS patients is unknown. However, PCOS is known to be associated with an increase in the generation of ROS by peripheral blood leukocytes [8]. In addition, PCOS subjects showed a reduction in the expression of genes involved in mitochondrial oxidative metabolism [9,23] and in

Table 3 – Odds ratios and 95% CIs for PCOS with log-transformed mtDNA copy number and metabolic factors

	Model 1[a]		Model 2[b]	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
mtDNA copy number	0.29 (0.13-0.67)	<.01	0.15 (0.04-0.56)	<.01
Systolic BP (mm Hg)	0.95 (0.91-1.00)	.06	0.96 (0.90-1.04)	.31
HOMA-IR	2.02 (1.03-3.96)	.04	4.26 (1.43-12.68)	<.01
TG (mmol/L)	1.01 (0.99-1.02)	.52	1.01 (0.99-1.03)	.51
HDL cholesterol (mmol/L)	1.00 (0.96-1.06)	.73	0.99 (0.94-1.07)	.98
SHBG (nmol/L)	1.01 (0.99-1.03)	.23	1.01 (0.99-1.03)	.23

[a] Model 1: unadjusted.
[b] Model 2: adjusted for age and BMI.

a regulatory coactivator of mitochondrial biogenesis, peroxisome proliferator–activated receptor-γ coactivator-1α [9]. For these reasons, we speculate that a close link exists between mtDNA copy number and the pathophysiology of PCOS, although the molecular pathway remains unclear at present. This hypothesis needs further exploration by longitudinal studies and an investigation of possible biologic mechanisms.

Recently, the role of the mtDNA in the risk for insulin resistance and diabetes has been explored using elegant molecular genetic transfer techniques in rats [24]; and the decreased mtDNA content in peripheral leukocytes were associated with the development of type 2 diabetes mellitus [25]. However, it is unclear so far whether mitochondrial abnormalities were a primary defect or occurred secondary to or in parallel with this insulin resistance. In our study, mtDNA copy number negatively correlated with insulin levels and HOMA-IR in both PCOS patients and control subjects; therefore, the association between mtDNA copy number and insulin resistance indices does not represent a typical finding of PCOS status. In addition, our multivariate model shows that the association between PCOS and mtDNA copy number is independent from insulin resistance or other metabolic parameters. Further study is required to elucidate the association between mtDNA and PCOS independently of insulin resistance.

Some studies report that CRP levels are increased in women with PCOS [26,27], supporting the hypothesis that the risk of diabetes and cardiovascular diseases is increased by chronic inflammation in PCOS. However, the results of different studies are divergent and do not confirm this suspicion [28]. In our study, CRP levels did not differ between PCOS women and controls; and mtDNA copy number was not associated with increased CRP levels. One reason for this could be that the women with PCOS who participated in our study had a smaller risk of cardiovascular disease compared with other studies because they were relatively young and healthy. Another possible reason is that CRP might not have increased, as it is a substance that increases secondary to the increase of cytokine (interleukin-6) by reactive oxygen [29].

We note that our study had several limitations. Its cross-sectional nature did not allow for a determination of a cause-effect relationship, and it was also limited by small sample size. Further studies using methods that can precisely measure mitochondrial function, such as measuring membrane potential or measuring mitochondrial gene mutation, are needed to prove the cause-effect relationship.

Although skeletal muscle represents the criterion standard as far as a target tissue for studying mitochondria, we assessed peripheral blood leukocytes here instead of conducting muscle biopsies. However, peripheral blood could be obtained in a noninvasive manner; and alterations in mtDNA copy number in leukocytes have been shown to be related to deletions and mutations of mtDNA in skeletal muscle, thus serving as a biomarker for the pathogenesis and progression of disease [30,31].

In summary, we observed decreased mtDNA copy numbers in PCOS patients in relation to controls independently of insulin resistance or other metabolic factors. Although more studies with larger population samples are necessary to confirm our findings, this study provides further support for future research to clarify both the pathogenic and the predictive role of mtDNA content in PCOS as an early biomarker of the disease.

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