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Original Article

Circulating levels of Cyclophilin A in women with PCOS: correlation with clinical and biochemical parameters

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Abstract: Women with polycystic ovary syndrome (PCOS) are more likely to suffer from obesity, insulin resistance, and chronic low-grade inflammation than other women. Cyclophilin A (CyPA) is known as an inflammatory mediator that is secreted by various types of cells in response to inflammatory stimuli. Previous studies have shown that immunohistochemical expressions and/or circulating levels of CyPA are high in many diseases that cause inflammatory conditions in the body. This study aimed to evaluate serum levels of CyPA and their correlation with clinical and biochemical parameters of women with PCOS. In the study population, we analyzed 49 consecutive patients with PCOS and 30 age and body mass index (BMI)-matched non-PCOS healthy volunteers (Control group). PCOS was diagnosed using Rotterdam criteria. Serum CypA levels were measured using a CyPA ELISA Kit. The relationship between serum CyPA levels and the clinicopathological variables of PCOS were also evaluated. Average levels of CyPA were lower in PCOS subjects than the non-PCOS subjects (21.5±3.1 and 38.5±4.2, respectively, P=0.0015). Serum CyPA levels were significantly correlated with homeostasis model assessment-insulin resistance (HOMA-IR) and high sensitivity C-reactive protein (hsCRP) levels in the PCOS group (r=0.230, P=0.04 and r=0.302, P=0.006, respectively). There was no correlation between serum CyPA levels and other clinical and biochemical parameters. Our study demonstrates that patients with PCOS have lower circulating levels of CyPA than women with normal ovaries. Decreased CyPA levels may be related to increased insulin resistance in PCOS patients. Further research is needed to evaluate the association between CyPA and PCOS.

Keywords: Cyclophilin A, low-grade inflammation, hyperandrogenemia, insulin resistance

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common complex endocrine pathologies. It is characterized by oligo/anovulation, hormonal and/or clinical hyperandrogenism, and the appearance of polycystic ovaries on ultrasound [1]. It affects 6-14% of women in reproductive age, and long-term complications include type 2 diabetes mellitus, cardiovascular diseases (CVD), and infertility [2, 3]. Therefore, it is important to understand the molecular basis of the pathophysiology of this syndrome.

Previous studies have reported that various biomarker alterations are associated with low-grade inflammation, endothelial dysfunction, hyperandrogenemia, dyslipidemia, obesity, and insulin resistance in women with PCOS [4-6].

Cyclophilin A (CyPA), an 18 kDa protein, is a member of the immunophilin family. It is a well-known cellular protein that is present in various human tissue cells [7, 8]. Previous studies have shown that immunohistochemical expressions and/or circulating levels of CyPA are high in many disorders including diabetes mellitus, asthma, rheumatoid arthritis, abdominal aortic aneurism, CVD, and sepsis which couse inflammatory conditions in the body [9-14].

A recent study conducted by Satoh reported that plasma CyPA levels were significantly higher in patients with significant coronary stenosis than those without it. Moreover, they found that CyPA levels were correlated with a number of atherosclerotic risk factors associated with oxidative stress (OS) [11]. OS is generated by reactive oxygen species (ROS), which activate a pathway that induces CyPA secretion [15].

Table 1. Clinical, hormonal, and biochemical characteristics of PCOS and non-PCOS groups

Variables	PCOS (n=49)	Non-PCOS (n=30)	*P*-Value
Age (years)	25.2±0.61	26.4±0.91	0.247[a]
BMI (kg/m^2)	26.4 (17.5-43.8)	24.2 (18.5-36.5)	0.091[b]
WHR	0.79±0.01	0.74±0.01	0.001[a]
Hirsutism score	5 (1-14)	1 (0-4)	<0.001[b]
Parental history of DM (%)	23/49 (46%)	8/30 (26.6%)	0.120[c]
Parental history of CVD (%)	10/49 (20.4%)	10/30 (33.3%)	0.309[c]
Smoking (%)	11/49 (22.4%)	2/30 (6.6%)	0.127[c]
Menstrual Cycle length (day)	39 (27-65)	28 (24-33)	<0.001[b]
Systolic blood pressure (mmHg)	111 (89-160)	100 (92-121)	0.010[b]
Diastolic blood pressure (mmHg)	71 (61-114)	62 (52-85)	<0.001[b]
CyPA (ng/mL)	21.5±3.1	38.5±4.2	0.0015[a]
FSH (mIU/mL)	6.2±0.2	6.8±0.3	0.086[a]
LH (mIU/mL)	8.1±0.7	5.4±0.4	0.008[a]
E2 (pg/mL)	34.1±2.3	39.2±3.9	0.237[b]
Total Testosteron (ng/dL)	1.2 (0.04-4.7)	0.1 (0.07-0.3)	<0.001[b]
Triglyceride (mg/dL)	97 (46-538)	66.5 (44-109)	<0.001[b]
Total Cholesterol (mg/dL)	176 (116-263)	173 (102-226)	0.302[b]
LDL (mg/dL)	101 (45-185)	95 (39-138)	0.317[b]
HDL (mg/dL)	52 (27-86)	63 (36-82)	<0.001[b]
HOMA-IR	3.4 (0.6-39.7)	1.4 (0.5-5.4)	<0.001[b]
hsCRP	3.5 (0.2-33.0)	1.2 (0.2-9.8)	<0.001[b]

[a]Independent samples t-test, [b]Mann-Whitney test, [c]Chi-squared test. Values: mean ± SEM or median (min-max). Note: Clinical, hormonal, and biochemical characteristics of our PCOS and non-PCOS groups. In PCOS patient significantly higher WHR, hirsutism score, menstruel cycle lenght, sistolic and diastolic blood pressure, HOMA-IR and hsCRP levels than non-PCOS patients.

Secreted CyPA mediates cell proliferation and the migration of inflammatory cells in vascular smooth muscle cells (VSMCs) and endothelial cells [16, 17]. Furthermore, CyPA activates DNA synthesis, and it inhibits nitric oxide-induced apoptosis in VSMCs [18].

Women with PCOS are more likely than other women to have increased cardiovascular risk factors, such as hyperinsulinemia, abnormal plasma lipids, hypertension and endothelial dysfunction (ED), as well as increased levels of C-reactive protein (CRP), endothelin-1, and homocysteine [19]. Of the mentioned risk factors in PCOS, ED is crucial in the development of CVD. ED is caused by imbalance between the production and bioavailability of endothelium-dependent relaxing factors and endothelium constricting factors, characterized as OS, which is associated with increased ROS generation and decreased antioxidant concentration. ED contributes to the increased risk of atherosclerosis and CVD in insulin-resistant subjects with PCOS [20].

Therefore, in this study, we addressed whether CyPA is a potential biomarker for determining cardiovascular risks in patients with PCOS. This study aimed to evaluate serum CyPA levels and their correlation with clinical and biochemical parameters of women with PCOS.

Materials and methods

The investigation protocol was approved by the Ethics Committee of Balikesir University, and all subjects gave their informed consent before participating in the study. The study protocols were in accordance with Helsinki Committee requirements.

Subjects

A cross-sectional study was designed and conducted at Balikesir University, School of Medicine, Education and Research Hospital between January 2016 and December 2016 to evaluate the serum CyPA levels in women with PCOS. Eighty women (including 50 with PCOS and 30 age- and body mass index (BMI)-matched healthy volunteers) were included in the study population. All the women with PCOS were selected consecutively. After giving a blood sample, one of the participants in the PCOS group declared that she wanted to withdraw from the study. Thus, data of 79 participants (49 PCOS subjects and 30 with non-PCOS subject [control group]) were evaluated in this study.

Patients who had other disorders with clinical features similar to PCOS, such as Cushing's syndrome, congenital adrenal hyperplasia, and androgen-secreting tumors, were excluded from the study. Patients who had taken medi-

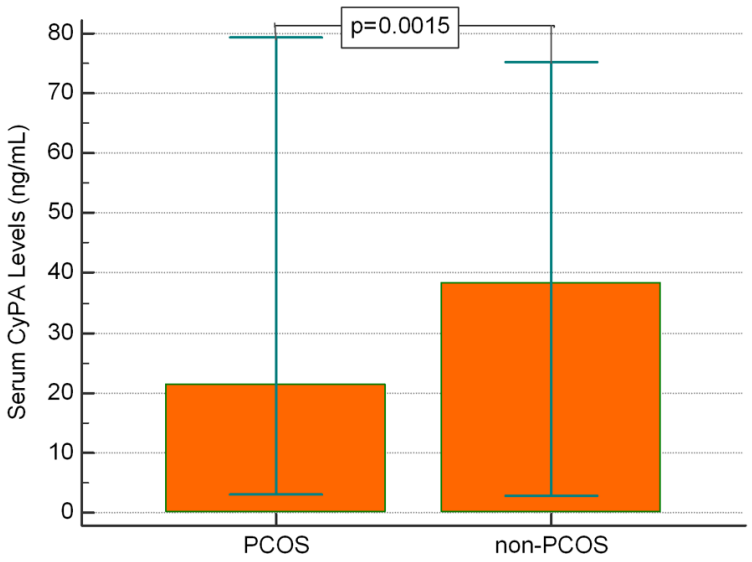

Figure 1. Average levels of serum CyPA of the two groups. Data represented lower average levels of CyPA in PCOS group than in non-PCOS group (P=0.0015).

cation for the previous 3 months, such as oral contraceptives, antilipidemic and/or antihypertensive medication, steroids, anti-diabetic medication, anticoagulants, or antiplatelet drugs, were also excluded from the study.

PCOS was diagnosed using Rotterdam criteria [1]. The presence of hirsutism was evaluated using the Ferriman-Gallwey scoring system [21]. A score equal to or greater than 8 was defined as hirsutism. Physical and gynecological examinations, ultrasonography monitorization, and peripheral venous blood sampling were performed during day 2 or day 3 of the study participants' menstrual cycles. After overnight fasting, blood samples were collected from an antecubital vein. Serum was collected after centrifugation at 2500×g for 10 min, and it was stored at -80°C until biochemical and hormonal assessment was undertaken.

Indices

Waist-to-hip ratio (WHR) was defined as the ratio of the waist measurement to the hip measurement. BMI was defined as the mass in kilograms divided by the square of the body height in meters (kg/m^2).

Biochemical evaluation

Serum CyPA levels were evaluated using commercially avaliable enzyme-linked immunosorbent assay kits (Sunredbio, Shangai, China). Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), thyroid-stimulating hormone (TSH), and total testosterone were determined using commercially avaliable enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, Austria) on a diagnostic instrument (BioTek, ELx 800, USA). Levels of glucose, total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol were measured using commercially available kits on a chemistry AutoAnalyzer (Cobas Integra 800; Roche Diagnostics GmbH; Mannheim, Germany). Serum C-reactive protein (CRP) was measured with chemiluminescent immunoassay using an ADVIA Centaur XP (Siemens Healthcare Diagnostics, NY, USA). Levels of fasting insulin were determined using commercial kits and an automatic hormone analyzer (Beckman Coulter; Unicel DXI 600; Access Immunoassay System). Homeostasis model assessment for insulin resistance (HOMA-IR) was defined as (insulin x glucose)/405.

Statistical analysis

MedCalc Statistical Software Program version 17.2 (MedCalc, Belgium) was used for the statistical analysis. The distribution of all variables in both the PCOS and non-PCOS groups was studied by describing the mean, median, range, and standard error of mean. The CyPA levels in the two groups were compared using the independent samples t-test. Receiver operator characteristic (ROC) curves were plotted to detect a cut-off value for the serum levels of CyPA levels to predict the levels for patients in both the PCOS and non-PCOS groups. A *P*-value of <0.05 was considered to be statistically significant.

Results

A total of 49 women with PCOS and 30 women without PCOS were included in the study. The clinical, hormonal, and biochemical characteristics of the women in the PCOS and non-PCOS groups are summarized in **Table 1**.

Table 2. Correlation between serum levels of CypA and clinical, biochemical and hormonal variables of patients with and without PCOS

Variables	PCOS CyPA	Non-PCOS CyPA
Age (years)	0.145 P=0.3202	0.103 P=0.5877
BMI (kg/m^2)	-0.0564 P=0.7001	0.165 P=0.3825
WHR	-0.0276 P=0.8506	0.0607 P=0.7500
Hirsutism score	0.148 P=0.3094	0.0909 P=0.6327
Systolic blood pressure (mmHg)	0.362 P=0.0105	0.0808 P=0.6713
Diastolic blood pressure (mmHg)	0.173 P=0.2356	0.0802 P=0.6734
Total testosterone (ng/dL)	0.154 P=0.2921	0.253 P=0.1779
Triglyceride (mg/dL)	-0.114 P=0.4337	0.207 P=0.2713
Total cholesterol (mg/dL)	-0.189 P=0.1925	0.0129 P=0.9460
LDL (mg/dL)	-0.176 P=0.2272	-0.103 P=0.5863
HDL (mg/dL)	0.0605 P=0.6798	-0.266 P=0.1547
HOMA-IR	0.520 P=0.0001	0.148 P=0.4337
hsCRP	0.479 P=0.0005	0.208 P=0.2708

Spearman's rank correlation.

There was no difference in age and BMI (P=0.247 and P=0.091, respectively) between the two groups. Parental history of DM and CVD and smoking rates were similar between the women in the PCOS and non-PCOS groups (P=0.120, P=0.309, and P=0.127, respectively).

Clinically, PCOS is characterized by an increase in WHR, a prolonged duration of the menstrual cycle and high blood pressure

WHR and hirsutism scores were significantly higher in the PCOS group than the non-PCOS group (P=0.001, and P<0.001, respectively). Menstrual cycle length was longer in the PCOS group than the non-PCOS group (P<0.001). Median systolic and diastolic blood pressures were higher in the PCOS group than the non-PCOS group (P=0.010, and P≤0.001, respectively).

Serum levels of CyPA were higher in PCOS patients

As shown in **Table 1**, the serum CyPA levels were significantly lower in the PCOS group than the non-PCOS group (21.5±3.1 and 38.5±4.2, respectively, P=0.0015) (**Figure 1**).

Biochemically, PCOS is characterized by increased insulin resistance, hyperandrogenemia, and low-grade inflammation

Regarding the other hormonal and biochemical parameters, median levels of total testosterone, triglyceride, HOMA-IR and high sensitivity C-reactive protein (hsCRP) were significantly higher in the PCOS group than the non-PCOS group (P<0.001, P<0.001, P<0.001 and P<0.001, respectively). However, the median levels of HDL were significantly lower in the PCOS group (P<0.001). Serum levels of FSH, E2, total cholesterol, and LDL were similar between the two groups.

Serum levels of CyPA strongly correlated with presence of hypertension, insulin resistance and inflammation in PCOS patients

The information presented in **Table 2** shows that the serum CyPA levels were significantly correlated with systolic blood pressure, the HOMA-IR and hsCRP levels in the PCOS group (P=0.0105, P=0.0001 and P=0.0005, respectively). There was no correlation between the serum CyPA levels and the other clinical and biochemical parameters in PCOS group. Also, there was no correlation between serum CyPA levels and clinical, biochemical and hormonal variables in non-PCOS group (**Table 2**).

The cut-off value of CyPA was 16.02 ng/mL for the prediction of PCOS in our study population

ROC curves were plotted to detect a cut-off value for the serum CyPA levels to the predict levels in the PCOS and non-PCOS groups. The ROC analysis demonstrated that the cut-off value of the serum CyPA levels was 16.02 ng/mL with a sensitivity of 63.3% and a specificity of 80% (**Figure 2**). The majority of women with PCOS had serum CyPA levels below this cut-off

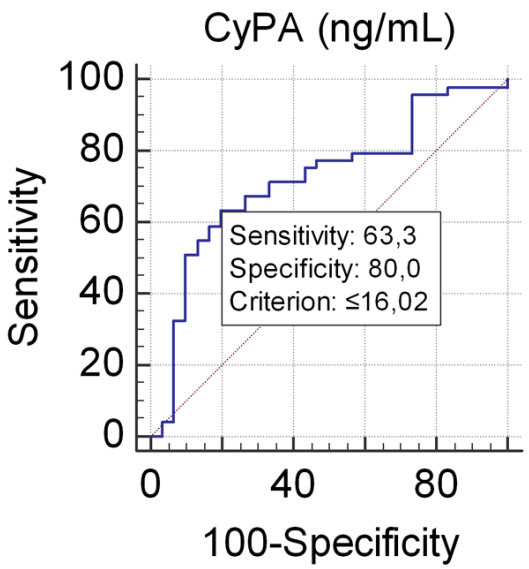

Figure 2. ROC analysis of study population. ROC analysis demonstrated that the cut-off value of the serum CyPA levels was 16.02 ng/mL with the sensitivity of 63.3% and specificity of 80%.

point. However, it was observed that the distribution range of the serum CyPA levels was wide. Some of the patients in the PCOS group had extremely high CyPA levels, but the only common common characteristics they shared were insulin resistance and high CRP levels.

Discussion

In the present study, we evaluated clinical and biochemical variables in PCOS subjects and compared them with age- and BMI-matched non-PCOS subjects. Our clinical and biochemical parameters confirmed that women with PCOS have higher triglyceride, HOMA-IR, total testosterone, and hsCRP levels, higher systolic and diastolic blood pressure readings, higher WHR and lower HDL values. We also found that serum CyPA levels were significantly lower in the PCOS group than the non-PCOS group. Also, correlation analysis showed that there was a positive correlation between serum CyPA levels and systolic blood pressure, HOMA-IR and hsCRP levels in patients with PCOS. To our knowledge, this is the first study to examine circulating levels of CyPA in women with PCOS.

PCOS is a complex endocrine and metabolic disease associated with obesity, insulin resistance and compensatory hyperinsulinemia, and chronic low-grade inflammation [22, 23]. Various biomarker alterations are associated with insulin resistance and low-grade inflammation in PCOS [4-6]. Insulin resistance and compensatory hyperinsulinemia in PCOS seem to have a stimulatory effect on chronic low-grade inflammation. Recent studies have also shown that obesity is associated with insulin rezistance and low-grade inflammation in PCOS [24].

CyPA, a multifunctional protein, is known to be an inflammatory mediator that is secreted from various types of cells in response to inflammatory stimuli, such as hypoxia, OS and infection, and many previous studies have shown that expression and/or circulating levels of CyPA are higher in disorders related to inflammatory conditions [12, 16, 18, 25]. Billich reported that CyPA levels increased in the synovial fluids of patients with rheumatoid arthritis (RA) [10]. Tegeder reported that CyPA activity was significantly higher in patients with severe sepsis than in healthy subjects [9]. Stemmy showed higher concentrations of extracellular CyPA in the chronic phase of asthma using a murine model [13]. Ramachandra reported that in comparison to the non-diabetic population, patients with type 2 diabetes mellitus have higher circulating levels of CyPA [12]. Yan reported that serum CyPA concentrations in unstable angina and acute myocardial infarction subjects were significantly higher than those in patients with stable angina and in the controls [26]. A recent study by Nigro found that CyPA expression was significantly higher in the atherosclerotic plaque of the arterial wall, and they concluded that CyPA is an inflammatory mediator that promotes atherosclerosis [14].

In the present study, contrary to the initial expectation, we found lower CyPA levels in the PCOS subject than non-PCOS subjects. This result may be due to differences in complex molecular mechanisms, as well as the severity of the inflammation in different inflammatory conditions. In most previous studies, increased levels of CyPA were found in severe inflammatory conditions, such as RA, sepsis, asthma, type 2 diabetes mellitus, unstable angina and acute myocardial infarction [9-14]. In contrast, the results of the study showed that women in the PCOS group had relatively low grade inflammation accompanied by insulin resistance and

mild hsCRP elevation and these results are compatible the findings reported in previous PCOS studies [22, 23]. Furthermore, we found that serum CyPA levels were positively correlated with systolic blood pressure, the HOMA-IR and hsCRP levels in patients with PCOS. These results indicate serum CyPA levels tend to elevate with increased severity of inflammatory conditions in patients with PCOS.

Additionally, differences between the sociodemographic features, such as age, gender, and BMI, and different sample sizes of studied populations are potential confounding factors that can cause these conflicting results. For example, a study by Li reported that the expression of CyPA in skin tissue increased with aging [27]. Similarly, a study comparing young rats with older rats reported that, CyPA expression was significantly higher in older rats [28]. In our study, for the reduced possibility of errors, the women in the PCOS group and the non-PCOS group were matched according to age and BMI.

Low-grade inflammation in PCOS is associated with an increase in plasma levels of hsCRP [29, 30]. It is known that hsCRP is a simple inflammatory biomarker, and it reflects future CVD risk [31]. We found that hsCRP levels in the women in the PCOS group were significantly higher than the women in the non-PCOS group, and our current results correspond to the findings reported in previous studies [29, 30]. According to our results, the women in the PCOS group are more likely to have a future risk of CVD than the women in the non-PCOS group.

Previous studies have reported that the presence of dyslipidemia is the most common metabolic co-morbidity in women with PCOS, and a lower HDL cholesterol level is the most frequent lipid abnormality in PCOS patients [32]. Lower HDL poses a cardiac risk even if other cholesterol levels are normal. In the present study, women in the PCOS group had lower HDL cholesterol levels than women in the non-PCOS group. Our present results are consistent with the findings reported in previous studies [33]. HDL cholesterol is referred to as good cholesterol because HDL particles can prevent atherosclerosis of the walls of blood vessels. Based on our results, the women in the PCOS group are at high risk for future atherosclerotic disease.

Regarding hyperandrogenism and insulin resistance, we found that women in the PCOS group had higher total testosterone and HOMA-IR levels than women in the non-PCOS group. Recent reports have confirmed these results [6]. The mechanism underlying hyperandrogenism and insulin resistance in PCOS patients is associated with an abnormal activation of the ERK1/2 pathway [34]. Recent studies have reported an alteration in ERK1/2 pathway function in skeletal muscle cells, in granulosa cells, and in theca cells for women with PCOS. These findings implicate abnormally lower ERK1/2 pathway activation in the pathogenesis of insulin resistance and excessive ovarian androgen production in PCOS patients [34, 35].

Interestingly, an animal model study conducted by Satoh found that, after complete ligation of arteria carotis, the intimal, medial, and adventitial thickening were significantly lower in CyPA knockout ($CyPA^{-/-}$) mice than in wild-type (WT) mice and mice overexpressing CyPA specifically in VSMC (VSMC-Tg). They also found that ERK1/2 activation and Ki67 cells were significantly decreased in the $CyPA^{-/-}$ mice [36]. These studies indicate that lower CyPA levels have a protective effect against tissue damage in inflammatory conditions. Additionally, in our previous study, we found that there were no significant differences in carotis intima media thickness between the PCOS group and the control group as reported in other previous studies [37, 38]. These results might be associated with lower levels of CyPA.

During the inflammatory process, CyPA mediates cell proliferation and migration in VSMCs and adhesion molecule expression in endothelial cells [11, 15, 18]. This proliferation is accrued by activation of the ERK1/2 pathway [16]. Thus, CyPA can stimulate DNA synthesis, and it inhibits nitric oxide induced apoptosis in VSMCs [18]. In addition, previous tumor cell line and culture medium studies have shown that secreted CyPA poses mitogenic activity on tumor cells via CD147. It is the only known signaling receptor for secreted CyPA, and blocking the secreted CyPA/CD147 interaction by monoclonal antibody against CD147 significantly suppresses the effect of secreted CyPA on cell proliferation and ERK1/2 activation in cells. CyPA was found to have a positive correlation with the phosphorilation of ERK1/2, and knock-

down of CyPA inhibited phosphorylation of ERK1/2 in cells [39]. These results suggest that there may be a possible relationship between lower levels of CyPA and abnormal ERK1/2 activation. In light of these studies, we extended our research to investigate the interactions between CyPA/CD147 and ERK1/2 pathway in PCOS patients.

This present study has some limitations. It used a cross-sectional study design and it had a relatively small number of the participants. However, it is the first study to investigate the serum CyPA levels in patients with PCOS using a multivariable and age- and BMI-matched study design.

In conclusion, the results of the study have shown that women with PCOS have lower circulating levels of CyPA than women with normal functioning ovaries. The low serum CyPA levels in the women in the PCOS group suggest that there may be a possible defect in the production of CyPA within the patients' cell or secreted out of their cells. Decreased CyPA levels in PCOS may be associated with abnormal activation of ERK pathway. Clearly, further studies are necessary to test this hypothesis.

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Disclosure of conflict of interest

None.

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